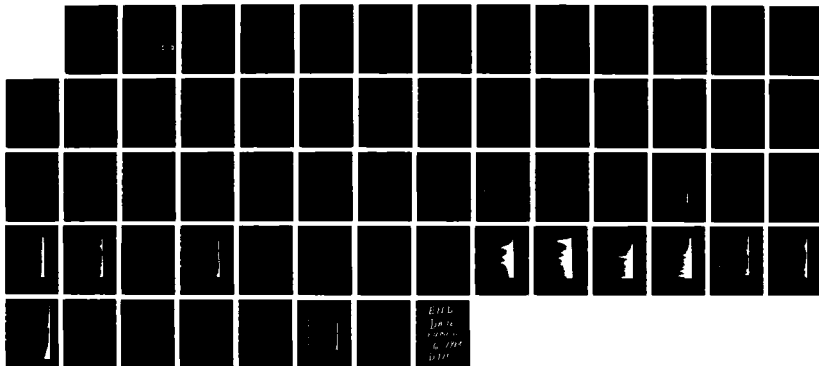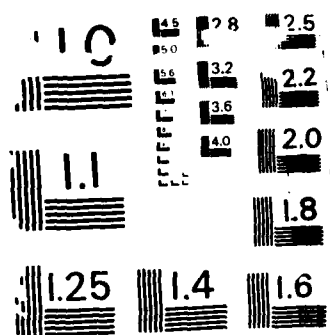CHEMICAL SYNTHESIS COXIELLA BURNETTI
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ANNUAL and FINAL REPORT

CHEMICAL SYNTHESIS <u>COXIELLA</u> <u>BURNETII</u> LIPOPOLYSACCHARIDES:

STRUCTURAL STUDIES OF <u>COXIELLA BURNETII</u> LIPOPOLYSACCHARIDES

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Lipopolysaccharide (LPS) materials were extracted from C. burnetii (CB9MI514 & CB9MIIC4) with a modified phenol-water procedure, purified by ultracentrifugation, and forwarded to this facility for structural characterization. The LPS was degraded by partial acid hydrolysis and separated into lipophilic and hydrophilic fractions with the designations, Lipid C and O-Antigen, respectively. Preliminary structural data has been obtained for Lipid C and the O-Antigen of CB9MI514 and more confirmatory data and corroborative evidence is provided for the structure of O-Antigen as well as new information on the Lipid C of CB9MIIC4. Sample permethylation on intact LPS (CB9MIIC4) by differential CD_3/CH_3 analysis has provided an approximation of molecular weight. Supercritical fluid chromatography (SFC) and SFC-MS studies have been initiated for application to this problem.

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Structural Studies of <u>C. burnetii</u> Lipopolysaccharides

abstract

Lipopolysaccharide (LPS) materials were extracted from <u>C. burnetii</u> (CB9MI514 & CB9MIIC4) with a modified phenol-water procedure, purified by ultracentrifugation, and forwarded to this facility for structural characterization. The LPS was degraded by partial acid hydrolysis and separated into lipophilic and hydrophilic fractions with the designations, Lipid C and O-Antigen, respectively. Structural data is presented for the O-antigen and lipid-C components in both phase I and phase II <u>Coxiella burnetii</u> LPS with some understanding of the core region of the phase II LPS. This information has been achieved by a combination of chemical degradation, derivatization, separations by gas-liquid and high-performance liquid chromatography with structural determination using several adjunct techniques in mass spectrometry. Sample permethylation of intact LPS (CB9MIIC4) using CD_3I or CH_3I differential analysis has provided an approximation of molecular weight. This final report summarizes the progress on lipopolysaccharide characterization and the analytical techniques developed to better amplify and augment the approach to this unique bacterial cell-surface material. In addition, we have briefly discussed the preliminary work done with immunological modifiers which have been partially purified from chloroform/methanol extracts and our contribution to the structural determination of compound"X"isolated from phase I. To be assured we were applying the most recent advances in analytical instrumentation, we have selected and are currently evaluating two instrumental techniques which seem highly appropriate to this LPS project, supercritical fluid chromatography (SFC) and capillary column zonal electrophoresis (CZE). These new developments are discussed in this report, other methods and procedures introduced for this LPS study have been described in our first annual report and are not repeated here.

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A. INTRODUCTION AND BACKGROUND

1. Introduction

Coxiella burnetii toxicity has been associated with the LPS of the virulent phase I cells. Phase II cells, which are avirulent, arise under conditions of laboratory cultivation and potentially during natural infections. The LPS of these latter organisms appears to lack the extended polysaccharide sequences associated with the phase I cells and may be analogous to the S-R variation in other forms of enteric bacilli.[1] Restriction analysis of the isogenic pair, (CB9MIC7 & CB9MIIC7), has revealed a missing fragment of chromosomal DNA in the phase II strain.[2] Restriction mapping and hybridization has provided evidence that the deletion is an 18 kb[3] fragment. A similar deletion of chromosomal DNA was shown to be missing from another independently derived intrastrain isolate (CB9MI514). This most interesting observation, along with chemical composition, PAGE patterns, and related phenotypes[4,5] indicates a structural complexity intermediate between phase I and II. In preliminary tests, CB9MI514 has proven to be as virulent as the CB9MI parent strain.[5] As pointed out by these latter authors, virulence (e.g., suppression of lymphocyte proliferation) may be associated with a rather small structural change in the LPS, 'semi-rough' to 'smooth', or in chemical terms extending the oligosaccharide beyond the phase II structure to include the additional structural components exhibited by CB9MI514. The relationship of this "intermediate" strain, with structural correlations to both I and II, combined with the interesting pathophysiological differences has provided a direct avenue for comparative structure-function evaluation. An identification of this biologically active entity, followed by component modification and/or chemical synthesis affords a direct focus to subunit vaccine development and provides the basis for this investigation. This report will summarize the structural evidence

obtained to date on the two phases of <u>Coxiella burnetii</u> and the methods and techniques by which this has been achieved.

2. Background

The results presented in this report have been based, in large part, on procedures developed specifically to enhance sensitivity and specificity for LPS analysis. With the exception of three recently introduced methods and techniques, we have not described in this report the analytical approaches utilized; these have been detailed in our first annual report.[6]

A summary of phase II LPS data indicates the molecule to be rather small, (<5,000 Da), consisting only of mannose and mannoheptose in the O-antigen with a very simple lipophilic portion containing principally 3-hydroxy C-15 fatty acids. The structural features of the core and Lipid-C region are incomplete. Phase I LPS has identical components in both the O-Antigen and Lipid-C regions and these details are discussed below in the appropriate sections.

B. NEW INSTRUMENTAL TECHNIQUES AND METHODS OF APPROACH

1. Supercritical Fluid Chromatography (SFC).

We are evaluating SFC because of its great potential for assisting in a solution of LPS structures. This potential is related to two factors: (i) its reported ability to provide chromatographic resolution (at ambient temperatures) for high molecular weight compounds; and, (ii) the potential for improved detectability on MS interfacing, (SFC-MS). The instrumentation has been provided by Lee Scientific and VG Analytical at no cost to this project.

Supercritical fluid chromatography employs a mobile phase consisting of a highly compressed gas near or above its critical temperature and pressure.[7,8] Under these conditions the

physical properties of the mobile phase merge to provide enhanced density (vis-a-vis gases) for improved analyte solubility and diminished viscosity (vis-a-vis liquids) with decreased resistance to mass transfer. When compared to HPLC, these features provide a greater number of effective plates or higher separation speed. Moreover, low temperature elution has been extremely successful in extending the thermal limits imposed by gas chromatography (GC) for the separation of thermally unstable and/or involatile materials. The recent introduction of capillary column SFC[9-11] provides improved efficiency with the advantages of diminished flow rates more appropriate to MS interfacing.[12-14]

With the earlier prospect of separating immunomodulatory components and application to Lipid-C purification, we chose to evaluate SFC using permethylated glycosphingolipids which would provide compounds of comparable polarity in plentiful supply. To date our studies of this technique have proved most encouraging and these results have been summarized and submitted for publication to Analytical Biochemistry, (a preprint of this paper is provided in the Appendix, section K. reference #5.). Split-injection is the current operating mode and we are making design modifications for a more sensitive "on column" injector. When these adjustments are in place we will initiate and apply SFC to the study of Lipid-C and O-antigen components. Although the details of instrument operation and glycolipid application can be found in the Appendix, we have included in the body of this report (Fig. 1), the separation of permethylated glucose polymers. Detectability is by flame-ionization. The high molecular weights resolved in this capillary column technique combined with the overall purification has proven to be most encouraging and we are enthusiastic about its application to the problems of LPS structure.

2. SFC and Mass Spectrometry Interfacing.

To maintain the high pressures within the column during SFC, a restriction of column effluent is required. We have

evaluated three different devices with which to obtain these high
pressures; a laser drilled disc restrictor, a polymer frit
restrictor, and a taper-drawn restrictor (Guthrie[15]). All three
devices are satisfactory for SFC when dectection is by flame-
ionization. Only the latter Guthrie restrictor, however, has
proven successful for SFC-MS interfacing. This "point" restrictor
provides rapid decompression into the vacuum of the MS ionization
chamber for sample desolvation and ionization before condensation
or precipitation and is undergoing further evaluation. In Figure
2 is presented a total-ionization plot obtained from an SFC-MS
analysis of 50 ng of a pemethylated pyridinylamine derivatized
sample of maltose. The chemical ionization mass spectrum, as
expected, provided a single ion for a determination of the sample
molecular weight, (Fig. 3). More recently we have seen molecular
ions for compounds exceeding 1,000 Da.

3. Capillary Column Zonal Electrophoresis (CZE).

CZE is a new powerful separations technique that has
recently been introduced. This simple apparatus combines the use
of capillary columns with extremely high voltage currents for the
high-performance electrophoretic separation of complex mixtures,
comparable to those observed in Coxiella burnetii extracts and
LPS hydrolysates. The methodology yields extremely sharp peaks,
with bandwidths equivalent to 10^5-10^6 theoretical plates per
meter, or greater. The high efficiency is, in part, a consequence
of the uniform osmotic flow profile across the capillary as well
as the high electrical potential. The separation occurs in a
capillary tube filled with a buffer and immersed in reservoirs at
each end. Electroosmotic flow is caused by the migration of ions
from the diffusive layer of the electrical double layer at the
capillary surface, under the influence of an electric field
imposed tangentially to the surface, causing the ions to migrate
toward the oppositely charged electrode. The resulting bulk
electroosmotic flow can be sufficiently fast so that positively
charged ions, neutrals species, and negatively charged ions elute

in short times, with the separation due to differences in the electrophoretic mobilities of the analytes.

Following these preliminary observations we, in collaboration with Waters Associates and Professor Harry Rubenstein (Dr. Rubenstein is spending a sabbatical year with us from Lowell University), have been investigating this chromatographic approach for oligosaccharides. For the oligosaccharides, a pyridinyl amine group was introduced at the reducing end for fluorescence detection[16] and to provide these neutral materials with a charged functional group by amine protonation. Shown in Figure 4 is a chromatographic separation of the same glucose polymers used in the SFC study but these are not derivatized. The exceedingly high column resolution makes this technique of great value for the separation of the complex mixtures obtained from bacterial extracts and partial hydrolysates. The mass spectrometry interfacing of this technique through a specially designed probe which can provide fast atom bombardment ionization is currently being considered, (Dynamic FAB, VG Analytical).

4. Deuterium Labeling and Differential Analysis.

To ascertain component molecular weight and replaceable hydrogens, we have chosen to use differential permethylation (C^1H_3/C^2H_3) analysis in combination with fast atom bombardment mass spectrometry. The molecular weight for the permethylated **Coxiella burnetii**, phase II LPS, was determined in this manner and confirmed by comparing the results with the perdeuterated sample. The CD_3 induced mass shift of the molecular ion, compared to CH_3, indicates the number of methyl groups incorporated in the sample from which the molecular weight can be calculated. These conditions cause de-O-acetylation, and thus, the molecular weight determined will be less than that calculated, see discussion below.

C. RESULTS

1. Coxiella burnetii LPS, Phase II.

a.) Lipid-C: (Composition, Derivatization, GC-MS, MIKES and DCI Mass Spectral Analysis). The lipophilic anchor of the Phase II LPS was prepared by acetic acid hydrolysis and extraction of the dried residue into methylene chloride. This soluble material has been designated Lipid-C. In preparation for this structural investigation we studied numerous synthetic and natural (Salmonella) analogs of Lipid-A, and tried to anticipate the structural problems that may ensue with the tiny amounts of Lipid-C. A method was subsequently developed to determine each of the N-, and O-acyl groups of the lipophilic anchor by a combination of basic hydrolysis and positive ion FAB MS. Unfortunately, preparations of Lipid-C provided no FAB mass spectra. This we attribute to the greater lipophilicity of Lipid-C, a consequence that is known to diminish FAB ionization. Mass spectra could be obtained, however, by direct chemical ionization (DCI), a process ideally suited for lipid samples. The resulting spectra with methane and ammonia (Fig. 5) as a reagent gas indicated Lipid-C to be uncharacteristic. A close examination of this spectrum shows several fragments and mass intervals of considerable interest, especially those between m/z 276, 398 and 674, (ammonium adduct ion, +18 amu), and the intervals 84 and 122, as indicated below:

$$
\begin{array}{ccccc}
276 & 398 & 657 & 758 \\
\end{array}
$$

```
        276    398   657     758
          \\      \\    \\      \\
  -\-—A—-\-\-B—-\\—C—-\-\—d —-\-
        (276) \(122) \(276) \(84)
          \      \     \      \
           758 \ 482 \ 360 \ 84
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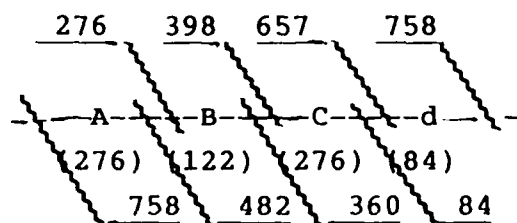
Although other variations of this fragmentation pattern can be ordered, these numerical relationships provide a model for

experimental testing. The 84 amu shift could imply an O-acyl ester equal to that mass (e.g., 276-->360, 398-->482, 674--> 758). The ion m/z 398 also represents the difference between two pairs of ions in the spectrum, (674 & 276, 758 & 360). And a loss of 276 amu from m/z 674 is equal to the fragment m/z 398. These relationships suggest a number of structural possibilities and with the accumulation of Lipid-C from CB9MI514, these possibilities will be tested. Methanolysis of this same fraction was undertaken to determine O-acyl fatty esters. The residue was dried and directly analyzed by DCI MS using ammonia as the reagent gas, (Fig. 6). Greater than 90% of the products could be accounted for as hydroxylated fatty acids and these results were supported by subsequent GC-MS studies following trimethylsilylation of the hydroxyl groups, (Fig. 7 and Table 1). Eight major hydroxylated fatty acids were identified, the most abundant proved to be 3-hydroxy-12-methyl tetradecanoic acid (3-OH, 12-Me, 14:0). It was not anticipated that hydroxy fatty esters would be detected following methanolysis[17] and this discrepancy must be considered further. These studies suggest Lipid-C to be an unusually low molecular weight molecule and the aliphatic character related primarily to hydroxy C-15 acids. An NMR analyis of Lipid-C showed no anomeric protons, suggesting an absence of carbohydrate. Acetylation of this lipophilic moiety was also investigated and a mass spectrum recorded. The hydroxylated fatty acid residues increased in mass by the expected 42 atm, but the area around what was anticipated to be the molecular ion region was of lower intensity and confusing. A new ion was observed at m/z 412; its relationship to the sample or other fragments is not clear.

To study in greater detail a mass attributed to the protonated molecular ion of Lipid-C, m/z 657, (DCI MS using CH_4 as reagent gas), a MIKES analysis was undertaken, (Fig. 8). This spectrum turned out to be very weak with many small fragments. However, one fragment of considerable interest is that of m/z 99 which is the mass equal to a protonated phosphoric acid moiety. It is surprising that other major fragments were not detected by

this method, but the data does suggest that a phosphate ester group is part of the m/z 657 fragment, the major ion in the Lipid-C extract.

Preliminary indications are that this phase II lipid anchor (Lipid-C) is similar in structure to that of phase I(514), (i.e., the lipophilic component provided a mass spectrum with many of these same ions, see below). Thus, we will continue to accumulate more material and acquire further data from the phase I sample.

b.) O-Antigen: (Component derivatization, Fluorescence HPLC, MIKES, FAB and DCI Spectral Analysis). The O-antigen of phase II is the residue left following mild acid hydrolysis and methylene chloride extraction. Direct analysis of this hydrolysate had proved unsuccessful and an interpretable spectrum was not obtained until the sample was per-O-acetylated. Examination of this residue by acetylation and FAB MS provided the spectrum in Figure 9, and these fragments can be rationalized to the structures in Table 2. Derivatization has subsequently proved to be most important to increase the detectability of all LPS products. The assignment of structure to the fragments in Figure 9 was helped considerably by subsequent ion collision and MIKES analysis of the daughter ions as presented in Figure 10. These fragments and their structural relationship to the parent tetrasaccharide, m/z 1339, has been detailed in Table 3. Further confirmation of the tetrasaccharide was achieved by treating the hydrolysate under conditions to fluorescently label glycose residues with pyridinylamine. The reaction mixture was purified by HPLC, (Fig. 11), which showed only a single peak. This fraction was isolated, permethylated and analyzed by FAB MS. The spectrum of the product obtained is presented in Figure 12 and shows a major ion, m/z 1027, (accounted for as the protonated molecular ion), and two minor fragments, m/z 823 & 809, which can be explained as terminal hexose losses, 204 & 219.

Much effort has been expended on alternative hydrolytic procedures and conditions in efforts to obtain larger

fragments than the tertasaccharide; fragments that would provide structural bridging pieces into the core region of the LPS. Milder hydrolytic conditions proved unsuccessful because of the very poor yield of any product. In struggling with this problem it was decided to complete confirmation of the tetrasaccharide with an alternative derivative. In this approach preparations of phase II were hydrolyzed, and derivatized by permethylation instead of acetylation. Permethylation is, in principle, less desirable because the basic conditions generated are known to cause de-O-acetylation. However, the FAB MS of this material provided ions that corroborate the tetrasaccharide structure, but surprisingly, several new, higher mass, ions were also obtained, (Fig. 13). Since permethylation would derivatize acidic functional groups, (phosphate, carboxylate), this approach, even though causing de-O-acetylation, appears to provide greater structural detail into the core region of the LPS. The higher mass ions observed in Figure 13 may reflect this fact. To understand the significance and relationship of these higher mass ions to the tetrasaccharide, each of the ions, (m/z 968, 1198, 1550, 1763), were separately mass focused, collided with helium and their daughter ion spectra obtained, (MIKES analysis, Fig. 14-17). The former three ions all gave rise to fragments indicating them to be composed of the tetrasaccharide, (e.g., m/z 468/436, 219/187), and thus, their important relationship to the LPS core region. The latter ion, m/z 1763, provided a daughter ion spectrum of considerable difference. Thus, our structural understanding has advanced beyond the tetrasaccharide to nearly twice the mass, 1700 Da. And considering the fact that these new structural pieces may have had O-acyl groups attached, that may mean we are half way to the molecular weight of the LPS, estimates to around 2800 Da, see below. Our current approach to capitalize on this new information is discussed below.

c.) **Estimation of Molecular Weight.** (Differential Derivatization, FAB & DCI MS). To obtain an approximation of phase II LPS molecular weight we have compared mass shifts

between permethylated and perdeuteriomethylated phase II LPS. The
two samples were analyzed by both FAB MS, (Fig. 18 and 19), and
DCI MS, (Fig. 20 and 21). FAB MS indicated the permethylated
sample to be 3375 Da and by a difference analysis with the
perdeuteriomethylation FAB mass spectral results, the derivative
contains approximately 40 methyl groups:

$$\frac{(3496) - (3376)}{3} = \frac{120}{3} = 40 \text{ methyls}$$

$$(3376) - (40 \times 14) = 2816 \text{ M.W.}$$

When the phase II LPS sample was analyzed by DCI MS the highest
ion was 2892 Da, suggesting a molecular weight approximating 2874
Da (subtraction of the ammonium adduct ion). This lower mass
value obtained for the permethylated derivative suggests sample
pyrolysis. This is not too surprising considering the process
must be initiated by probe heating. Comparison with the
perdeuteriomethylated sample indicates this fragment to have 32
methyl groups, leaving the lost fragment with 8 methyl groups.
Thus, there may be an unusually labile linkage between this
residue and the total molecular weight determined by FAB MS,
(3375 Da). An interesting spin-off from this LPS molecular weight
determination can be seen with a comparison of the low mass end
of the DCI spectrum, (500-2000 Da), to that obtained from partial
hydrolysis, permethylation and FAB MS. As might be expected the
pyrolytic fragments arising from DCI analysis of the intact LPS
molecule have their corollary in the partially hydrolyzed
permethylated products, (Fig. 13). Unfortunately, the FAB spectra
of intact LPS cannot be compared at low mass because they are
saturated under 2000 Da. The FAB MS data show the permethylated
hydrolytic fragments of m/z 968, 1198, 1550 1763, while the DCI
MS spectrum of intact permethylated LPS show pyrolytic fragments
of m/z 954, 1198, 1505, 1718, (Fig. 22). The mass differences

between the first and latter two fragments may be explained by the analysis method and their pyrolytic or hydrolytic origin. Further comparisons between the deuteriomethylated and methylated samples indicated the expected number of methyl groups, 15, into the tertasaccharide and 18 and 28 methyl groups into the fragment ions m/z 1198 and 1505, respectively. Although a skeleton of the core region appears to be taking shape through extensions of the hydrophilic O-antigen, no direct molecular entity has, as yet, put this puzzle together.

2. Coxiella burnetii 9MI514, Phase I & Compound X.

a.) Lipid C: (DCI Mass Spectrometry). Lipid-C has been prepared from CB9MI514 under conditions described for phase II. The dried residue was extracted with methylene chloride and this extract analyzed by ammonia DCI MS, (Fig. 23). The spectra for both extracts (phase II, Fig. 5; phase I, Fig. 23) show structural similarity with two groups of ions centered around m/z 276 and 674. This latter ion in the phase I appears as the MH$^+$ ion (m/z 657) and not the (M+NH$_4$)$^+$ adduct and may be the result of small pressure differences in the ion source of the mass spectrometer. There does appear to be real differences in lower molecular weight fatty acids that have accumulated in this phase I extract. With ions for the C-12 through C-16 fatty acids. Whether these additional acids are an integral part of the Lipid-C must await further experiments. There has always been spectral variation in the hydrolysate-methylene chloride extracts, and until a chromatographic system can be developed for component purification, it probably will continue. This we hope is where SFC would play a major role.

b.) O-Antigen: (Derivatization, DCI & FAB Mass Spectrometry). The residue following hydrolysis and methylene chloride extraction has been designated the O-antigen fraction. Direct analyses of these residues were unsuccessful and only after sample derivatization were spectra obtained. Phase I has

been prepared under the same procedures as phase II and FAB MS analysis provided only a pair of ions at m/z 2035 & 2019. DCI MS on the same sample, however, proved somewhat more successful, (Fig. 24). Most of the ions obtained from the DCI spectrum can be rationalized to a number of expected structures and this relationship is presented in Table 4.

c.) **Immunomodulatory Factors:** (Gas-Liquid Chromatography). Whole cell vaccines (WCV) from phase I strains are modulators of the immune response giving rise to immunity and the related adverse immunopathological reactions. These vaccines, when injected IP, gave rise to hepatomegaly, splenomegaly, liver necrosis, death, and supression of mitogenic and antigenic lymphocyte proliferation. Subfractions of these extracts have been shown to influence gross pathology and/or immune modulation in C57BL/10ScN endotoxin-nonresponder mice. The direct interest in C. burnetii subfractions relates to the hope of isolating components that elicit protective immunity separated from the tissue pathology. Such hopes were realized when components responsible for immune modulation were separated from whole cell vaccines (WCV) by chloroform-methanol extraction (CME) while the insoluble residues (CMR) still induced immunity.[18] In a subsequent study the immunomodulatory activities of WCV, CMRV, and CME were compared.[19] The CMRV did not induce either immunopathological reaction or supression of mitogenic and antigenic lymphocyte proliferation, in fact, in vitro lymphocyte proliferative responsiveness correlated with the protective efficacy of the vaccine. However, the deleterious tissue reactions and immunosupression did occur when the CMRV was recombined with the CME. Phase I LPS has been shown to be an important determinant of virulence, and this work[19] has shown that the presence of phase I LPS in CMRV does not induce the deleterious tissue reactions or immune suppression which suggests that other components are responsible for the immunopathology. Thus, fractionation of C. burnetii has provided a better understanding into the immunopathology of infection, which may

not solely rest with LPS I. Phase I cells must contain specific determinants of a suppressive complex which are dissociated by extraction with chloroform-methanol and the extracts must contain components which are required to activate the CMRV to produce an active suppressor complex. Selective CMR and CME fractions have been forwarded to this laboratory and we evaluated several techniques to isolate, purify, and perform structural analysis on the components of this mixture. Presented in Figures 25-28 are gas-liquid chromatograms of some of the extracts. DCI MS analysis of these fractions were unsuccessful in ascertaining differences probably because major components dominated the mass spectra and obliterated small differences. Although these gas chromatograms show excellent separations, it is important to note the limitations imposed by the temperature constraints of the column, sample and instrument. Many additional high molecular weight components are probably not eluted from this column. This is one of the reasons we became interested in SFC, where density programming initiates elution and temperature is not a limiting factor. Thus, high molecular weight separations can be achieved with the resolving power experienced with capillary columns. This project unfortunately will not be pursued during the forthcoming contract period.

d.) Compound X: (Derivatization, FAB & DCI Mass Spectrometry. Earlier work[7] on the phase I LPS had indicated a unique disaccharide and several other unidentified components. We, along with Dr. Williams' group (USAMRIID), shared in the structural characterization of this compound. The isolate was identified by DCI and FAB MS, (Fig. 29), using four different derivatives. These results are summarized in Scheme 1. Our efforts were related to derivative preparation and mass spectral analysis and interpretation. This data has subsequently been published in Journal of Biological Chemistry, 1987, (see Appendix, K. reference #3).

D. DISCUSSION OF RESULTS

1. Coxiella burnetii LPS Phase II.

Coxiella burnetii LPS appears to be composed of a unique lipophilic anchor with little structural analogy to the Lipid-A isolated from Salmonella. The chemical lability between this lipid anchor and the classical core and/or O-antigen poses a difficult structural problem in characterization of this bridging entity. This structural variation from the Salmonella LPS has required the development of new strategies to isolate and collect component pieces. The apparent absence of carbohydrate in the Lipid-C anchoring region requires new thinking about this lipid terminating group. We have not been successful in obtaining larger molecular weight fractions than those described and milder conditions of hydrolysis or weaker acids diminish the overall yield to undetectable levels. A similar problem has been experienced with the O-antigen. The current fragmentation pattern obtained from Lipid-C is most informative and with additional material many experiments can be undertaken to clarify these relationships. The preliminary results suggest an O-acyl group equal to 84 amu, a C-15 hydroxy acid conjugated to a base of molecular weight equal to 398 Da. It is not clear if this fragment 398 contains an additional fatty acid or not. The 122 amu interval provides some thought along this line but a reasonable structure has not been realized. One aliphatic tail does not seem sufficient for proper anchoring, but it is highly possible that some O-acyl esters have already been lost and the ion m/z 657 represents a partially stripped residue.

The structure of the O-antigen is slowly coming to light. The new bridging fragments obtained by permethylation may provide a back door to the core and Lipid-C structure. Most of these new fragments have provided daughter-ions indicating a tetrasaccharide relationship. With the understanding of a phosphate ester in one of the fragments, selective diazomethylation followed by acetylation would salvage O-acyl

groups and this may provide an approach to the current lability of the bridging region.

2. Coxiella burnetii LPS Phase I.

We have recently started structural investigations on CB9MI514. With these LPS samples our efforts can now focus on phase I. The data indicates a similarity in the Lipid-C moiety, and, this would be most fortunate. The tetrasaccharide is a major component of the O-antigen and the detection of compound X provides another approach to ferret out the LPS structure, (e.g., reductive methylation of these amine groups with formaldehyde & $NaBH_4$). Partial hydrolysis, fluorescent labeling, and HPLC has shown a series of eluting minor peaks suggesting a homopolymer array. Several runs will be necessary to acquire sufficient material for derivatization and MS analysis. A partially hydrolyzed sample, without fractionation has been sent to Dr. Williams (USAMRIID) for biological testing. If partial hydrolysis does not destroy the biological activity, we will follow our isolated fractions with biological testing and direct our efforts to those materials.

E. CONCLUSIONS

The primary objective of this study is to ascertain the structure-function relationships of LPS components to develop useful subunit vaccines. These results are a product of two research groups with major interests in the central components of this study, a pathological understanding of Coxiella infections, (USAMRIID) and the structural analysis of carbohydrate and glycoconjugate materials (Harvard U.). This report summarizes the progress of the latter group. The analytical techniques utilized represent a combination of methods developed specifically for this LPS study and an application of state-of-the-art instrumentation.

Although there is insufficient data to draw complete molecular structures, major conclusions can be reached about selected moieties in each, and important relationships between phase I and II can now be appreciated. Both _hase I and II appear to show similar terminating groups, (i.e., Lipid-C anchor and a tetrasaccharide residue at the opposing end). Recent data suggests that there is also similarity in portions of the intervening Core region and other data indicates a polymer series in phase I which was not detected in phase II. The further elucidation of these points and confirmation of reported structures will continue and the opportunity to cross-relate the two phases for new and different results represents a powerful comparative tool.

As a consequence of this investigation, and beyond the structural progress summarized in this report, four papers describing new methodology,[1-3,5] one paper describing structural results[4] (partially supported by this study), and four book chapters covering related analytical summaries[6-9] have been published, or, are in the process of being published.

F. RECOMMENDATIONS

We have great interest in continuing this project. The structural pursuit of these LPS molecules are intellectually as well as analytically challenging and bringing this project to fruition can provide a large measure of practical insight into gram negative sepsis and vaccine development. The anomalous structures realized from this study has demanded extra measures of methodological development and considerable alertness to subtle detail. This investment of effort we would wish to appreciate and capitalize on. Moreover, the dovetailing of our analytical instrumentation and glycoconjugate interests with Dr. Williams' efforts in providing LPS material make this collaboration not only focused but cost effective. Thus, for all these reasons we strongly recommend continuation of this Coxiella LPS project.

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H. LIST OF SCHEMES, TABLES AND FIGURES

I. PUBLICATIONS SUPPORTED BY CONTRACT

1. Her GR, Santikarn S, Reinhold VN, Williams JC, Simplified Approach to HPLC Precolumn Fluorescent Labeling of Carbohydrates: N-(2-Pyridinyl)-Glycosamines, Carbohydr Chem, 1987; 6:129-139.

2. Santikarn S, Her GR, Reinhold VN, Oligosaccharide Structural Studies By On-Line HPLC-MS Using Fast Atom Bombardment Ionization, Carbohydr Chem, 1987; 6:141-154.

3. J. Kieu, G.R. Her, and V.N. Reinhold. Supercritical Fluid Chromatography of Glycosphingolipids, Anal Biochem, 1988; (in press).

4. Amano K-I, Williams JC, Missler SR, Reinhold VN, Structure and Biological Relationships of Coxiella burnetii Lipopolysaccharides, J Biol Chem, 1987; 262:4740-4747.

5. S. Santikarn, V.N. Reinhold, C.E. Costello, and C.D. Warren, Determination of oligosaccharide branching by MS-MS. (manuscript in preparation).

BOOK REVIEWS AND CHAPTERS

6. Reinhold VN, High Molecular Weight Oligosaccharide Mass Spectrometry in 'Mass Spectrometry in Biomedical Research,' John Wiley & Sons, Simon J Gaskill, Ed, 1986, pp 181-213.

7. Reinhold VN, Oligosaccharide Chemical Ionization in Methods in Enzymology, Complex Carbohydrates, Part E. Vol 138, Victor Ginsburg Editor, Academic Press, Inc., 1987, pp 59-84.

8. Reinhold VN, Carbohydrate Mass Spectrometry. In: Glycoprotein Structure and Conformation; ed. R Ivatt, Plenum Press, New York, 1988, (in press).

9. Reinhold VN, Structural Analysis of Complex Carbohydrates by Ammonia Direct Chemical Ionization and Fast Atom Bombardment Mass Spectrometry in 'Molecular Immunology of Complex Carbohydrates' Plenum Publishing Corp, Albert M Wu, ed., 1988, (in press).

J. LIST OF PERSONNEL RECEIVING SUPPORT, (Two Year Period)

S. Santikarn, Ph.D. (23 Months)

G.R. Her, Ph.D. (24 Months)

V.N. Reinhold, Ph.D. (24 Months)

J. Kuei, Ph.D. (8 Months)

Ms. Domon (part time 3 Months)

Ms. S. Schulman (11 Months)

K. APPENDIX

1. Her GR, Santikarn S, Reinhold VN, Williams JC, Simplified Approach to HPLC Precolumn Fluorescent Labeling of Carbohydrates: N-(2-Pyridinyl)-Glycosamines, Carbohydr Chem, 1987; 6:129-139.

2. Santikarn S, Her GR, Reinhold VN, Oligosaccharide Structural Studies By On-Line HPLc-MS Using Fast Atom Bombardment Ionization, Carbohydr Chem, 1987; 6:141-154.

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4. J. Kieu, G.R. Her, and V.N. Reinhold. Supercritical Fluid Chromatography of Glycosphingolipids, Anal Biochem, 1988; (in press).

5. S. Santikarn, V.N. Reinhold, C.E. Costello, and C.D. Warren, Determination of oligosaccharide branching by MS-MS.

6. Reinhold VN, High Molecular Weight Oligosaccharide Mass Spectrometry in 'Mass Spectrometry in Biomedical Research,' John Wiley & Sons, Simon J Gaskill, Ed, 1986, pp 181-213.

7. Reinhold VN, Oligosaccharide Chemical Ionization in Methods in Enzymology, Complex Carbohydrates, Part E. Vol 138, Victor Ginsburg Editor, Academic Press, Inc., 1987, pp 59-84.

K. SCHEMES AND FIGURES   (PAGES 26 – 59)

STRUCTURE DETERMINATION OF COMPOUND X

Scheme 1

TABLE 1
FATTY ACID METHYL ESTERS

Scan Number	Structure
268	3-OH-12-Me-13:0
281	3-OH-14:0
303	3-OH-13-Me-14:0
306	3-OH-12-Me-14:0
316	3-OH-15:0
336	3-OH-14-Me-15:0
349	3-OH-16:0
372	3-OH-14-Me-16:0

page 28

TABLE 2

FAB-MS

FRAGMENT ION STRUCTURES AS DETERMINED BY FAST ATOM BOMBARDMENT MASS SPECTROMETRY

Fragment Ion	Structure
691
1051
1339
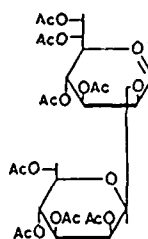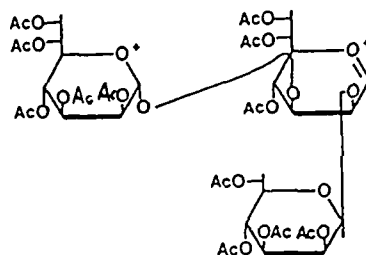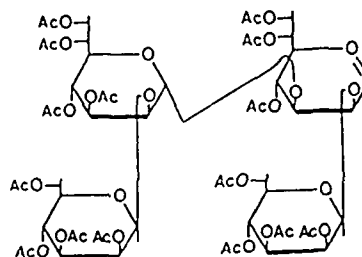
TABLE 3
CID/MS -MS

DAUGHTER IONS AS DETERMINED BY COLLISION INDUCED DISSOCIATION

Parent Ion ($^m/_z$)	Structure
1339	
991	
950	
691	
331	
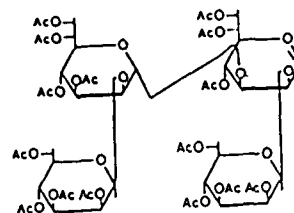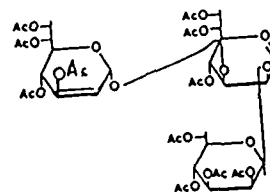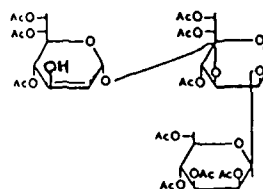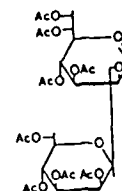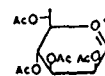
TABLE 4

DCI – MS

Fragment Ion	Structure
408	
480	
666	
1374	
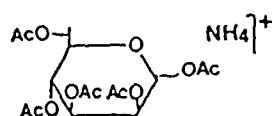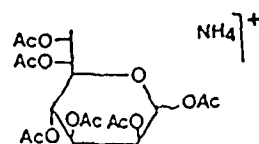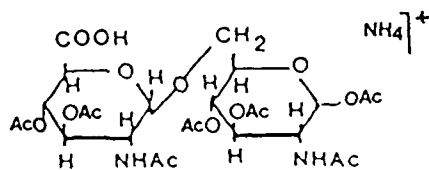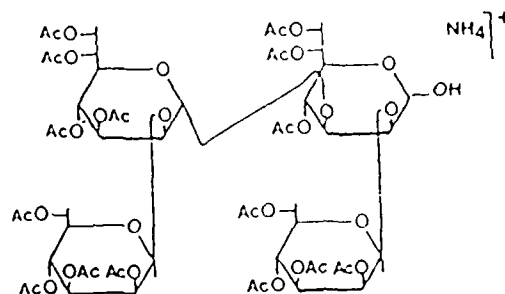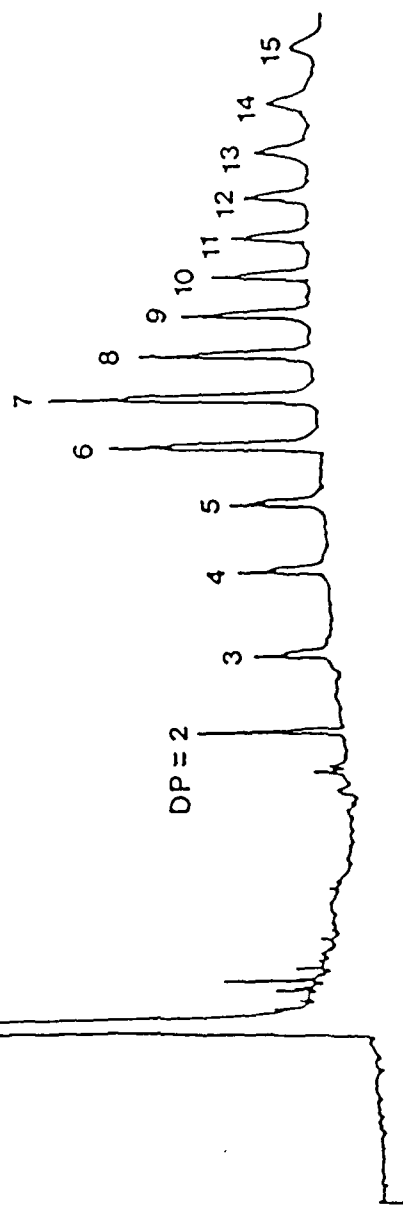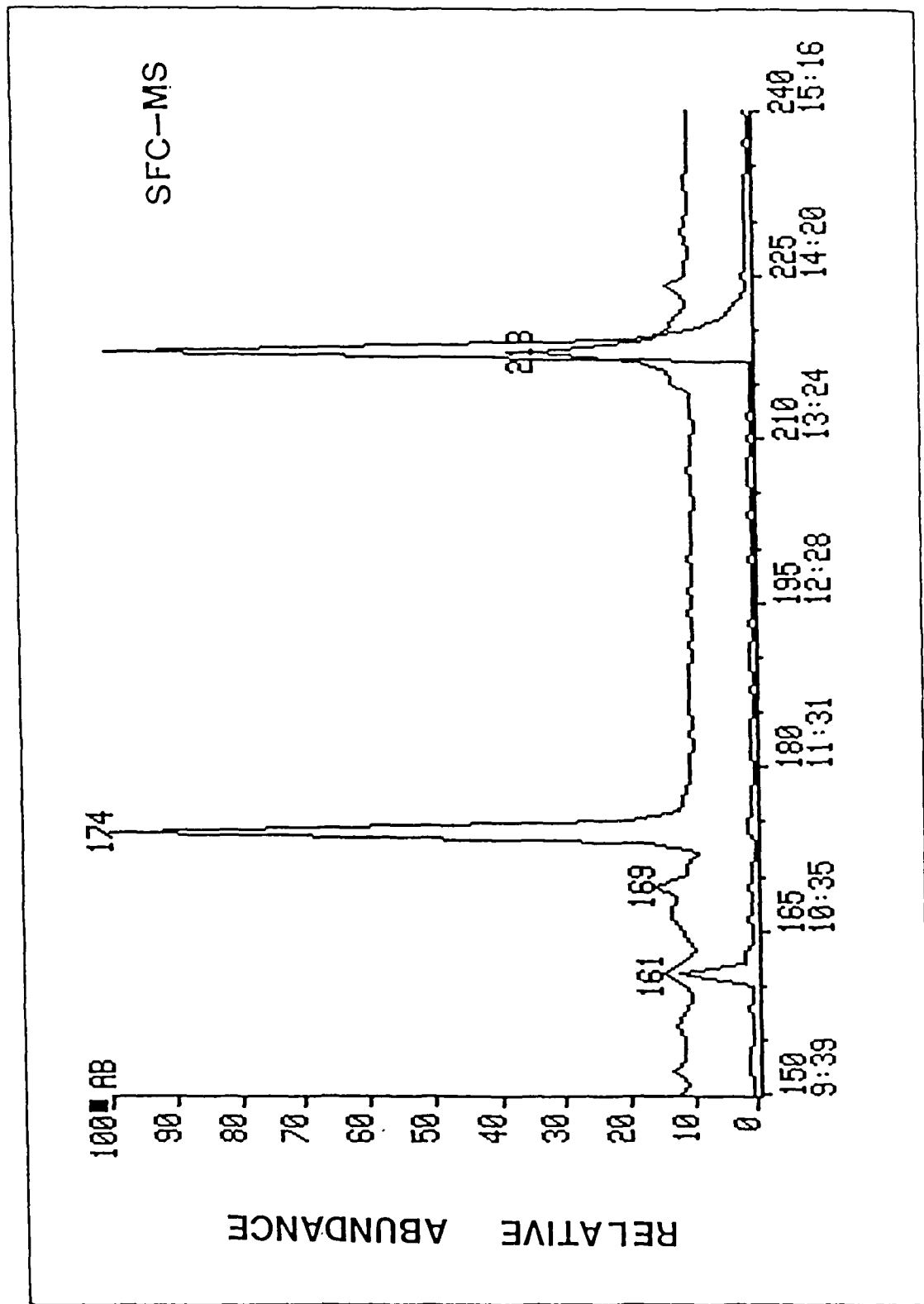
Figure 1

Figure 2

METHANOLYSIS LIPID C DCI-MS (NH3)
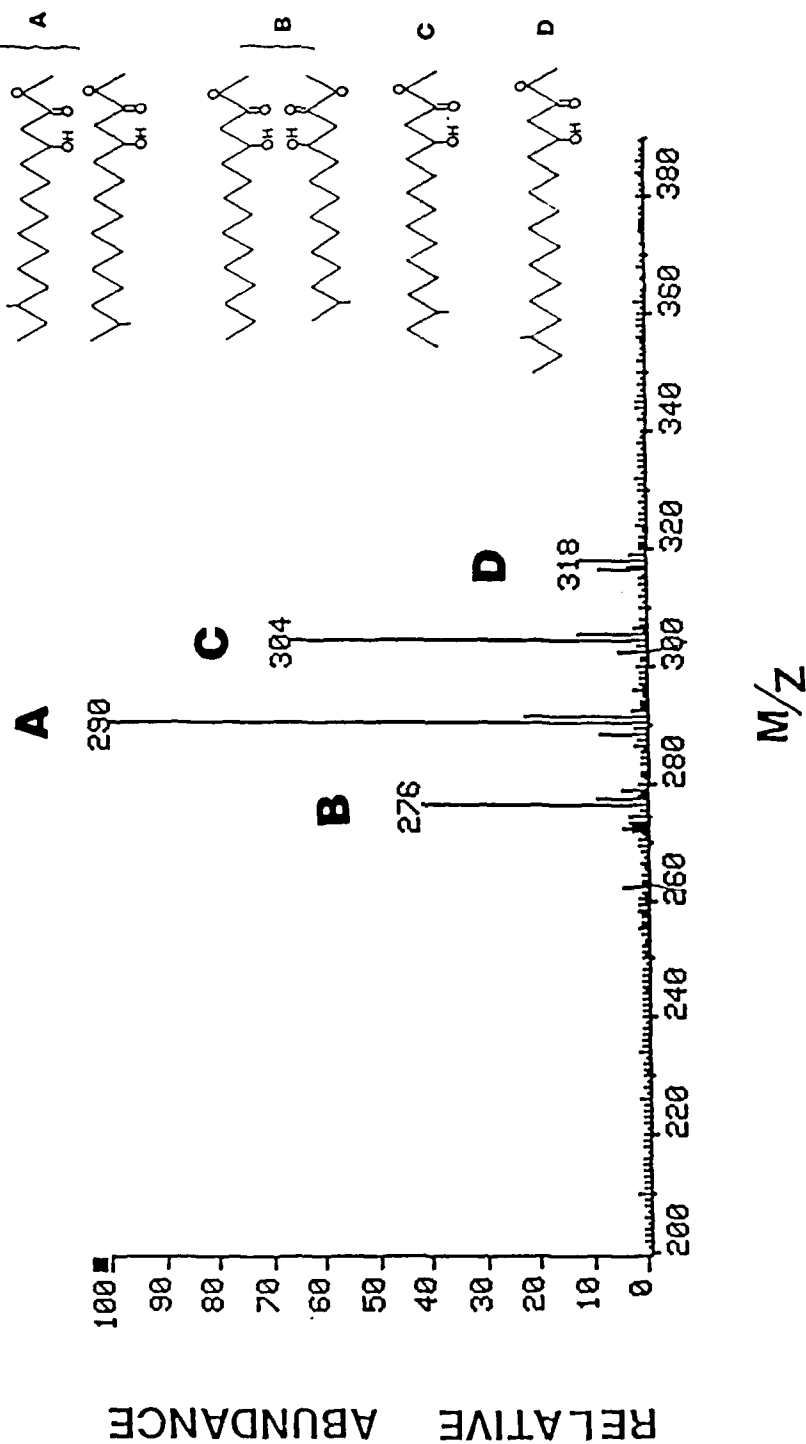
Figure 6

METHANOLISIS OF LIPID C , GC-MS

Figure 7

page 36
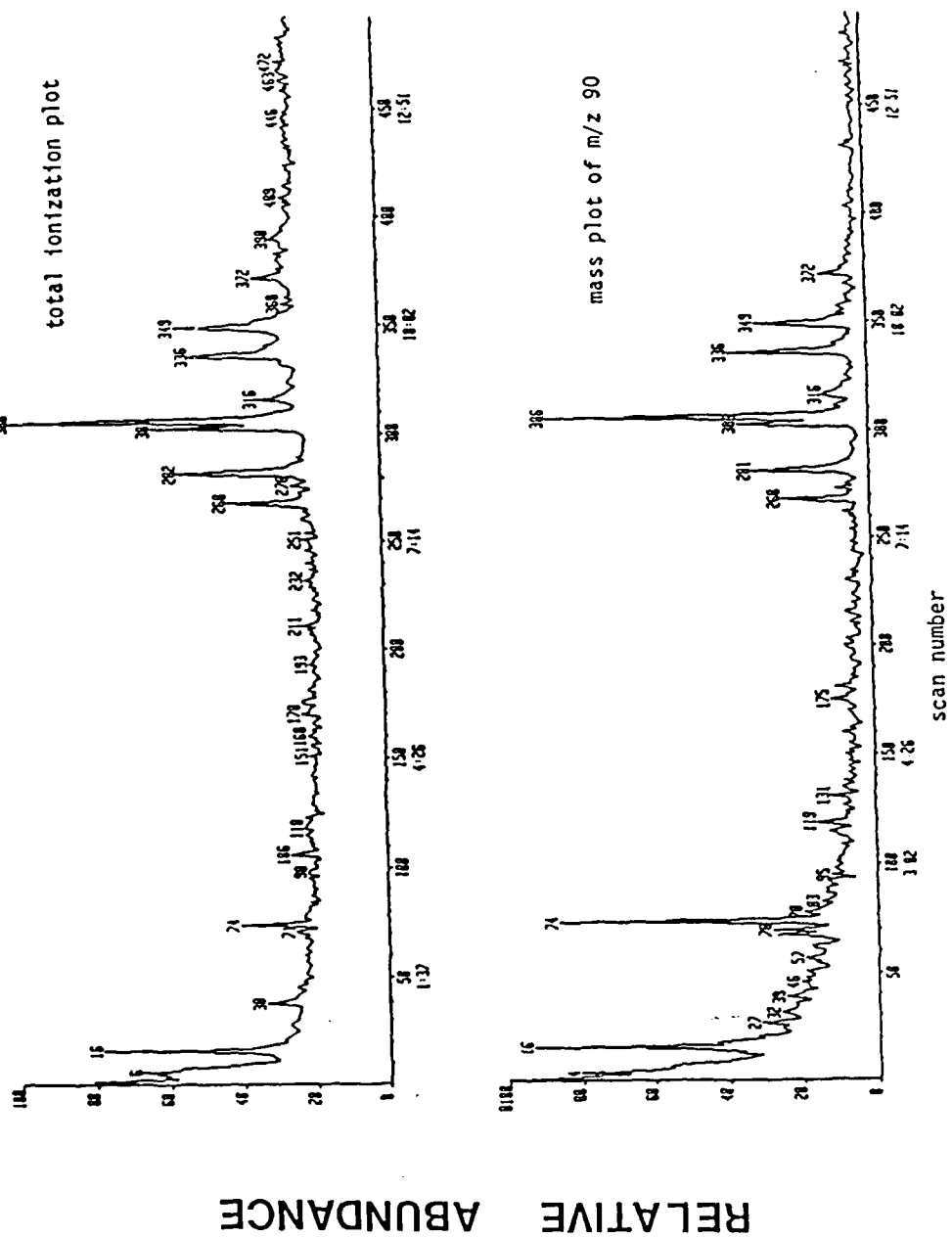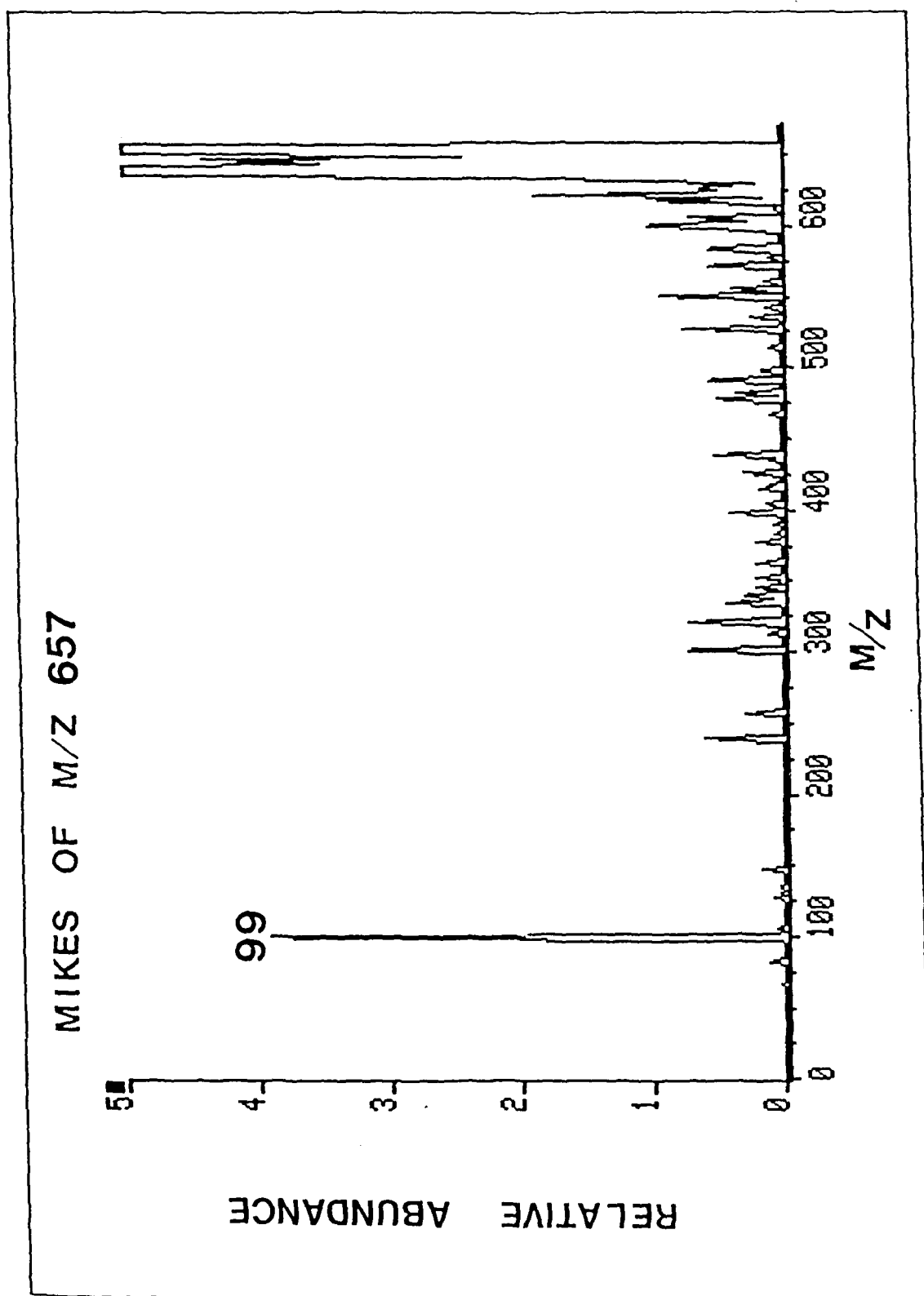
MIKES OF M/Z 657

Figure 8

FAB-MS PER-O-ACETYL O-ANTIGEN
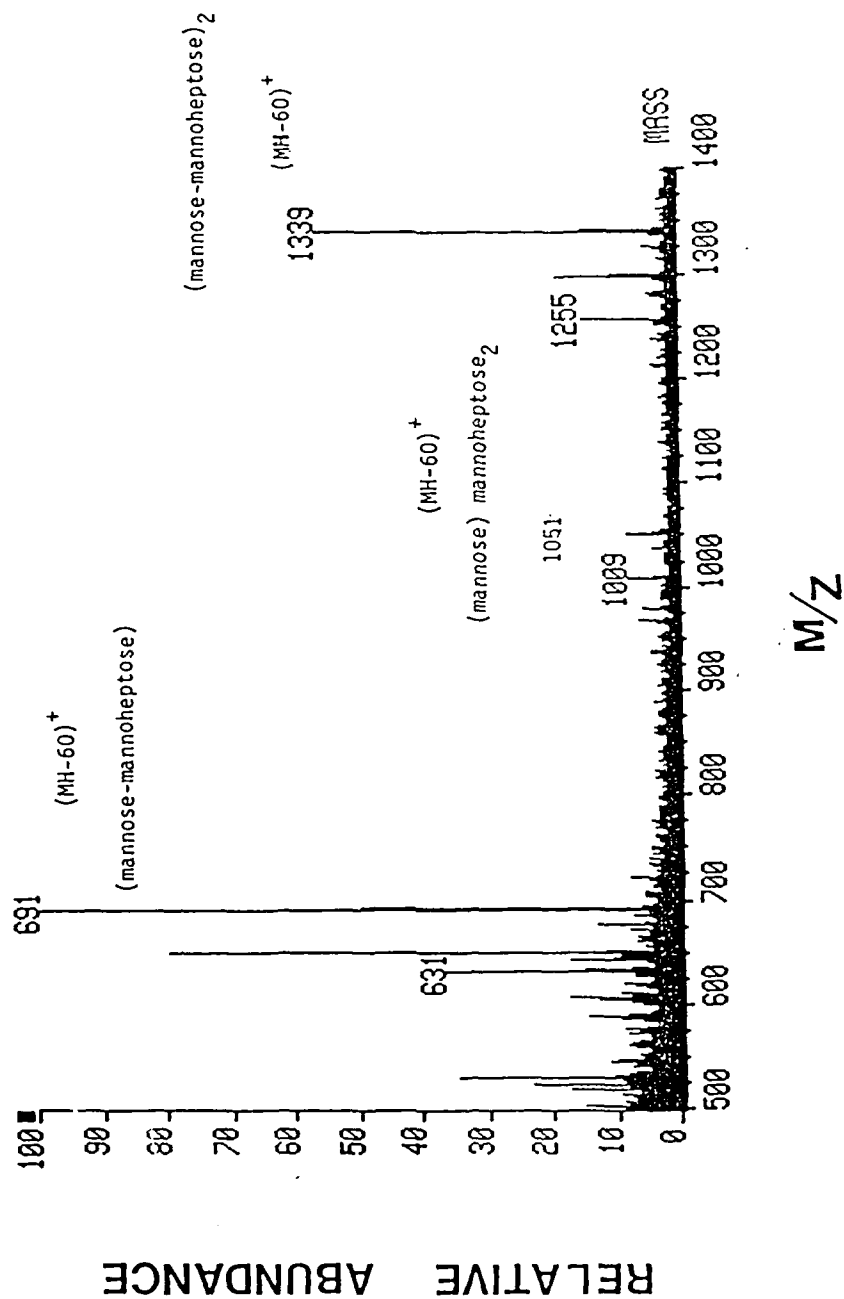
Figure 9

MIKES OF M/Z **1339**

Figure 10

HPLC

PA-TETRASACCHARIDE
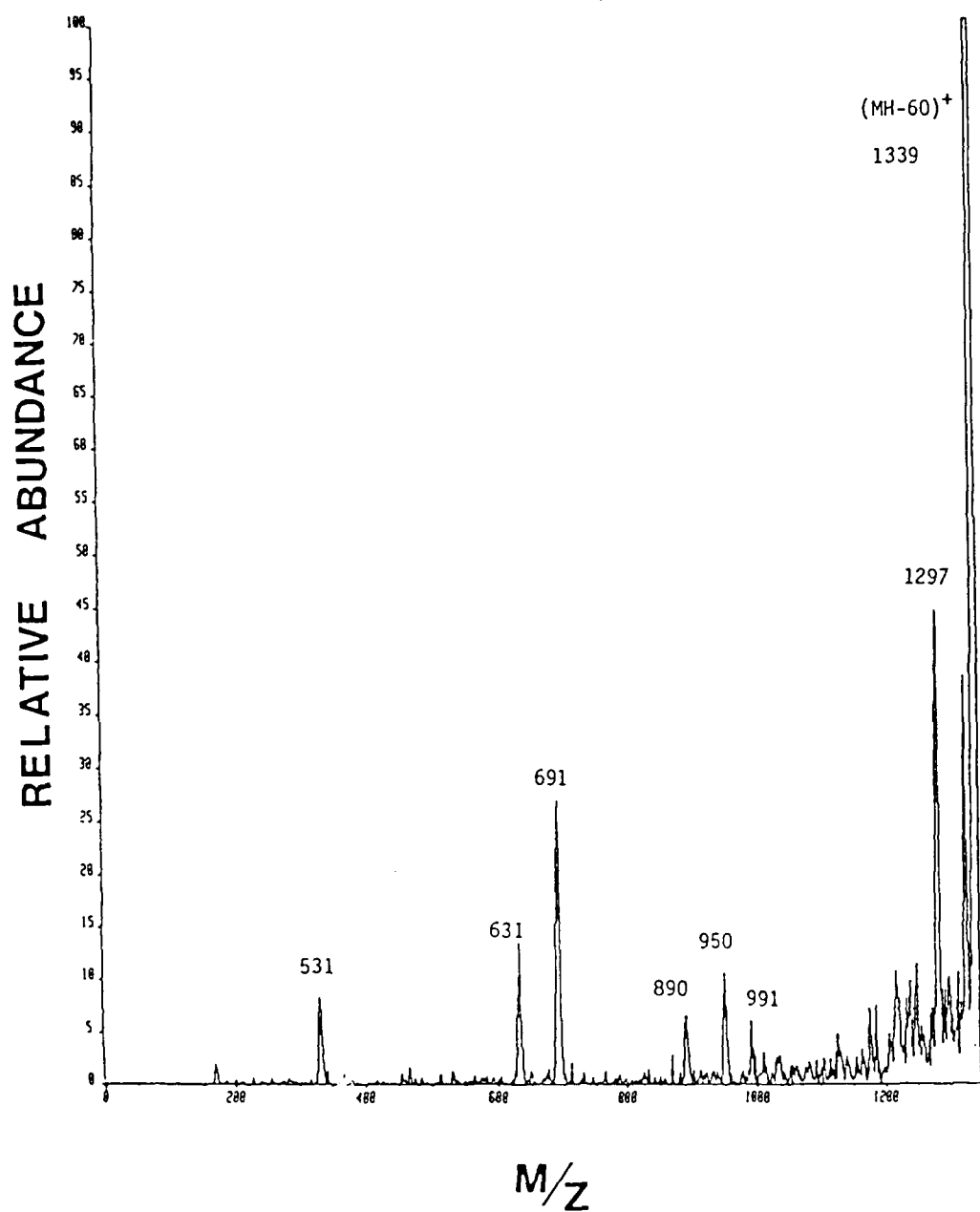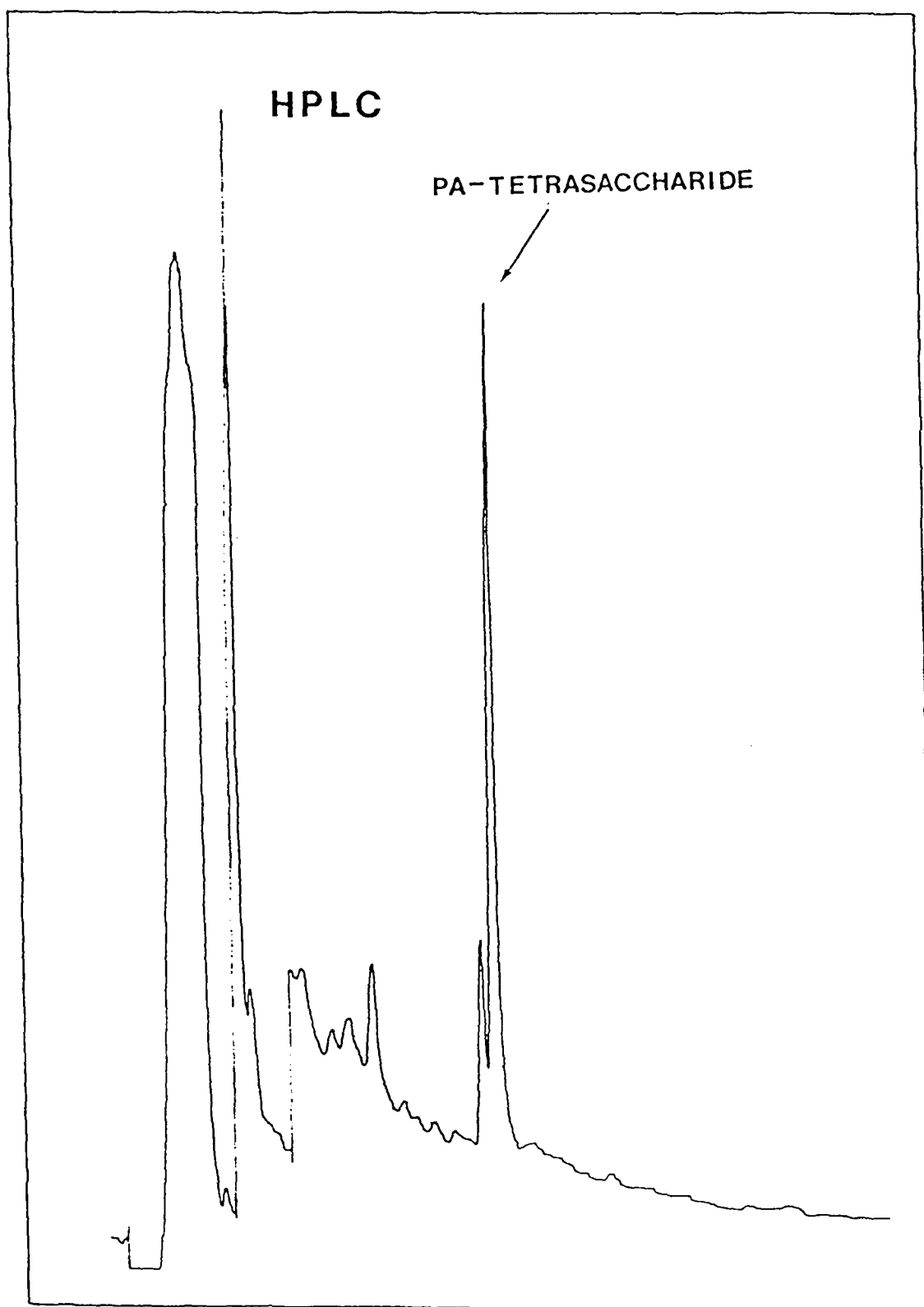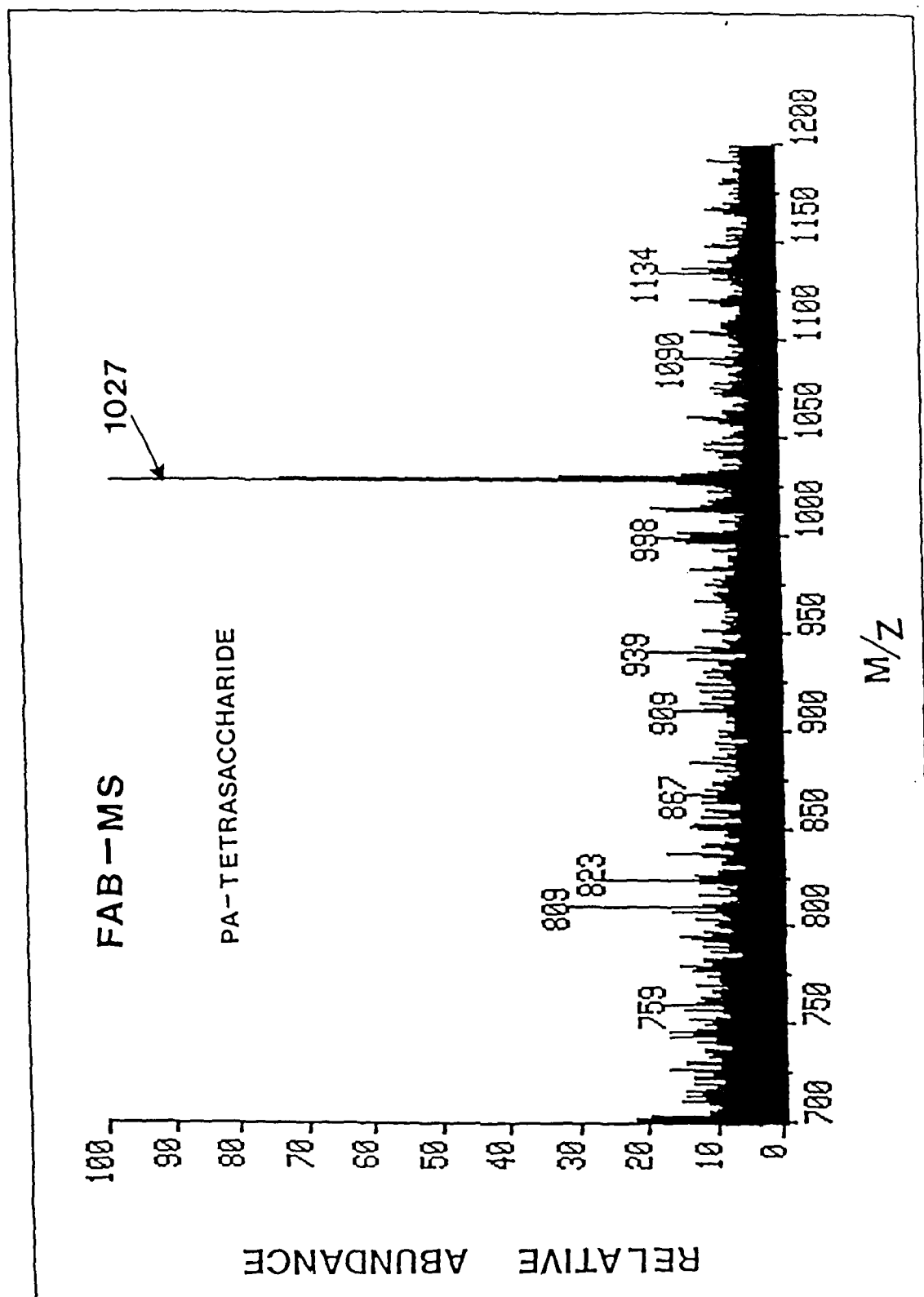
Figure 11

Figure 12
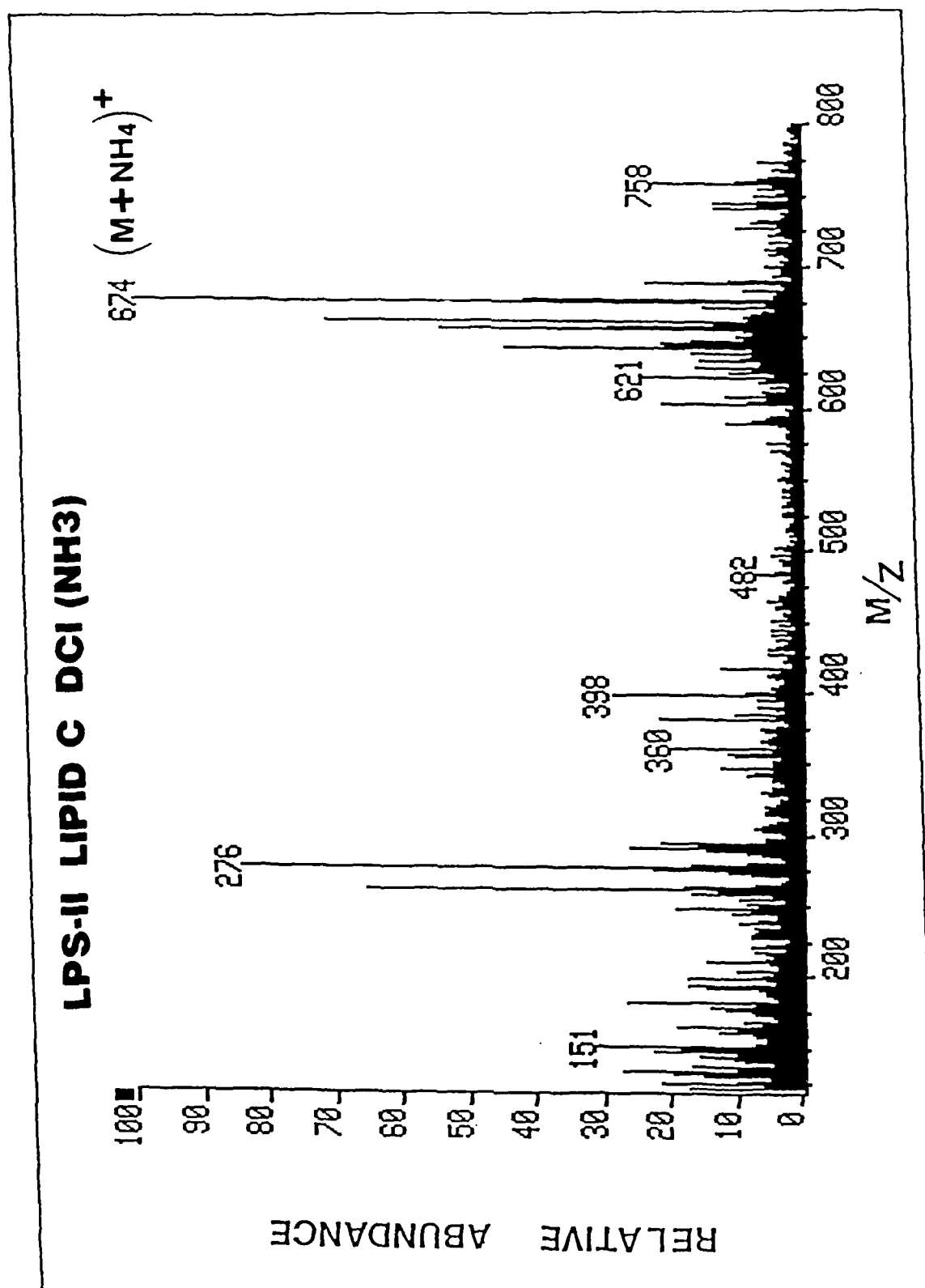
Figure 5

Figure 3

Figure 13

MIKES OF M/Z 968

Figure 14

MIKES OF M/Z 1198

Figure 15

MIKES OF M/Z 1550

Figure 16

MIKES OF M/Z 1763
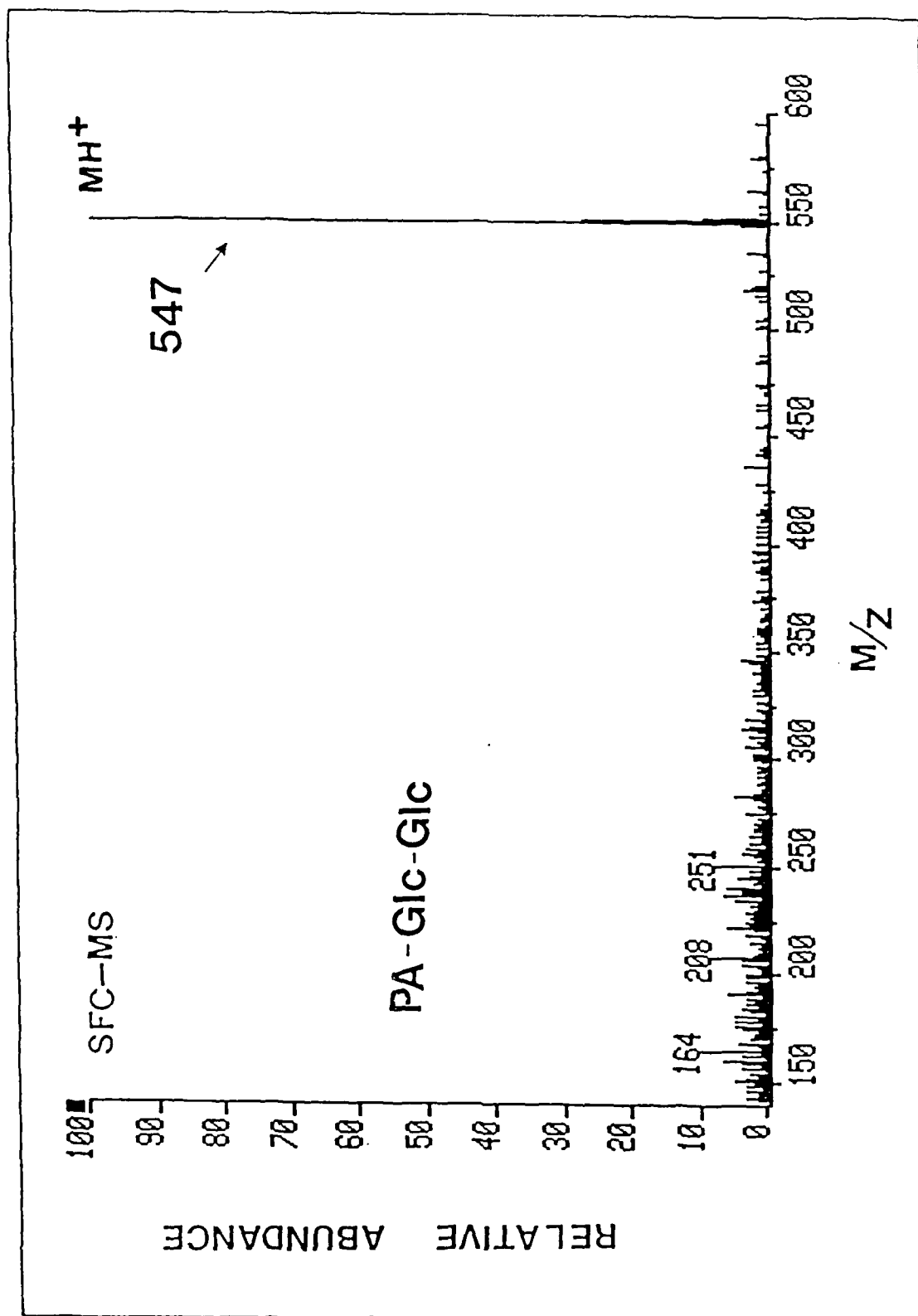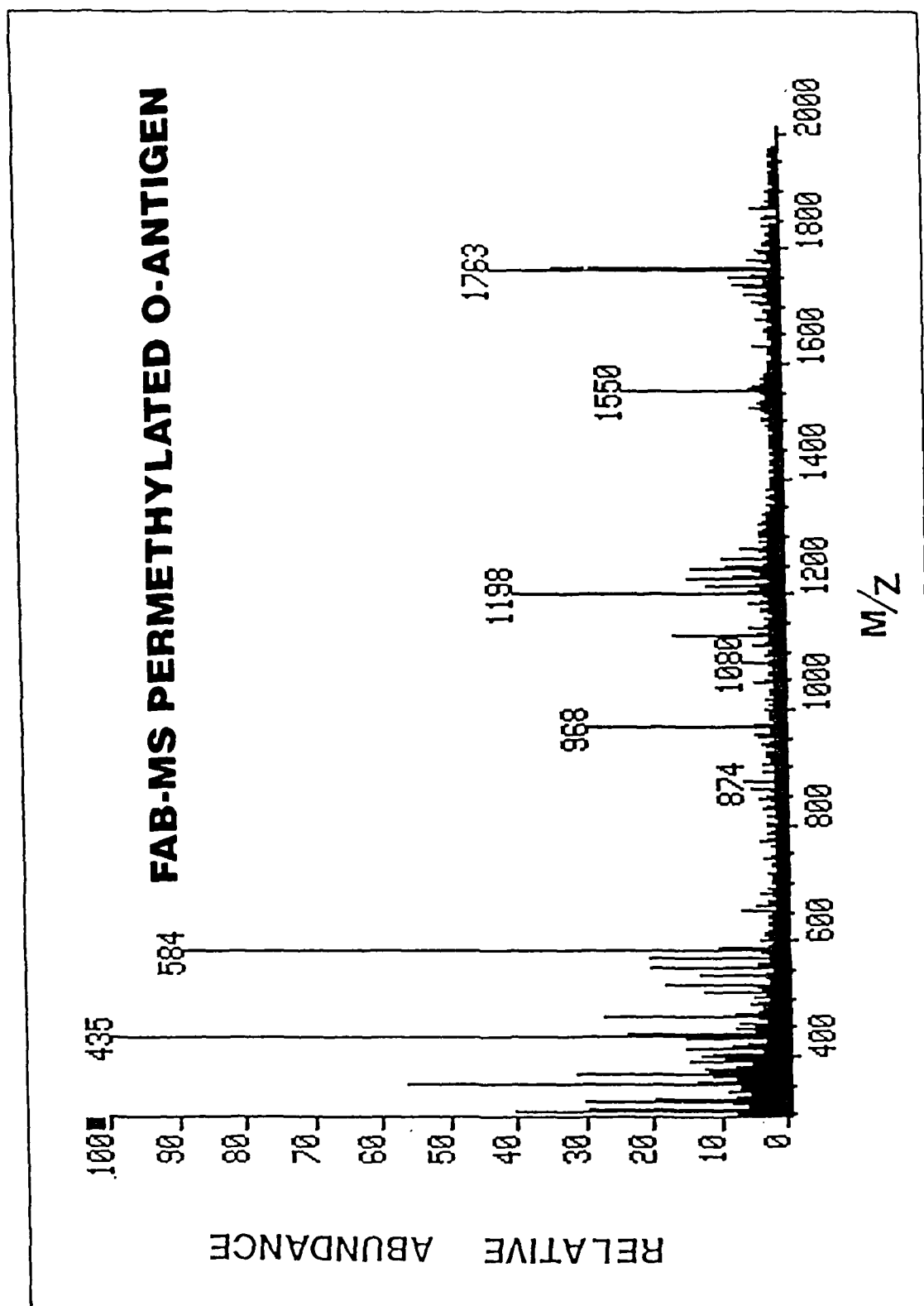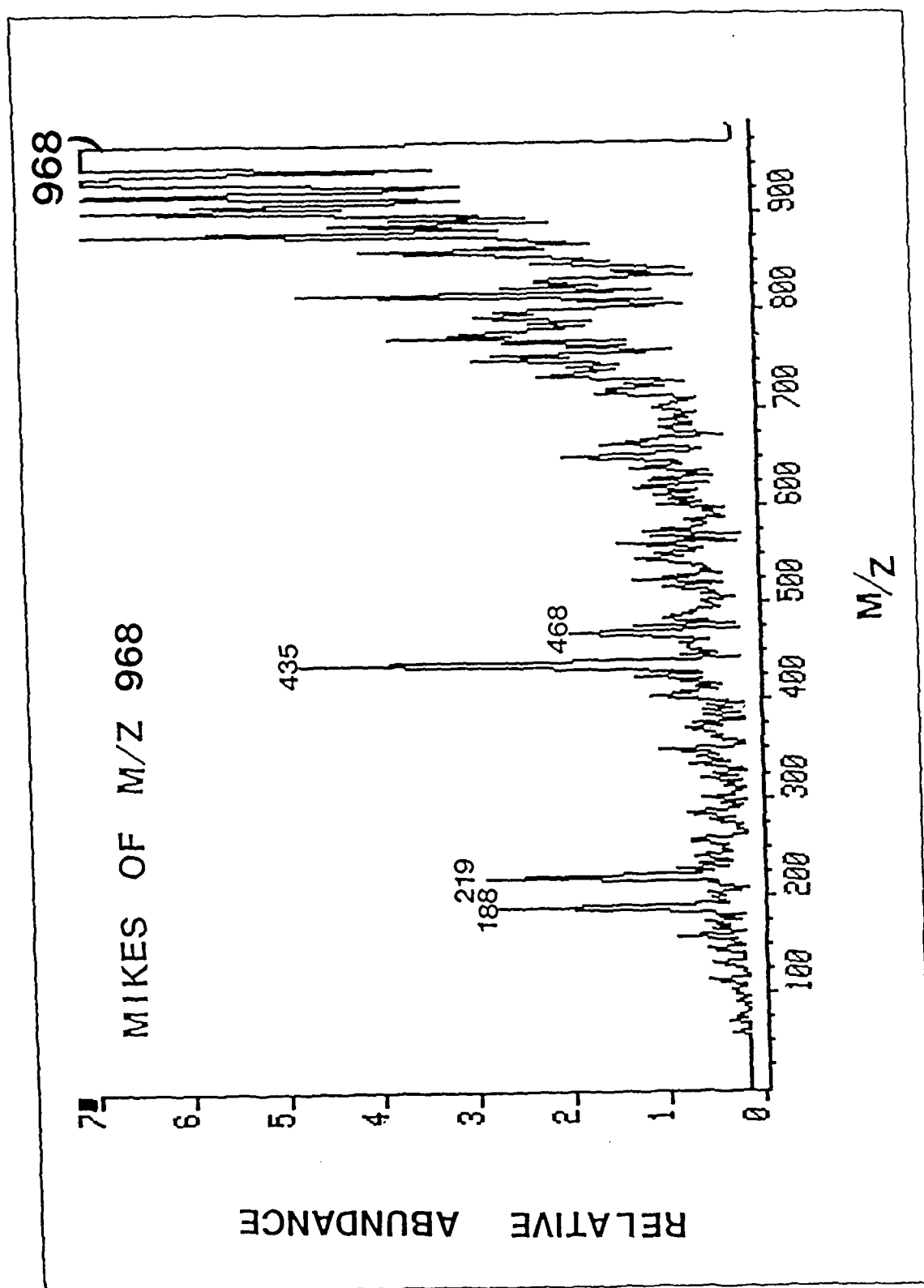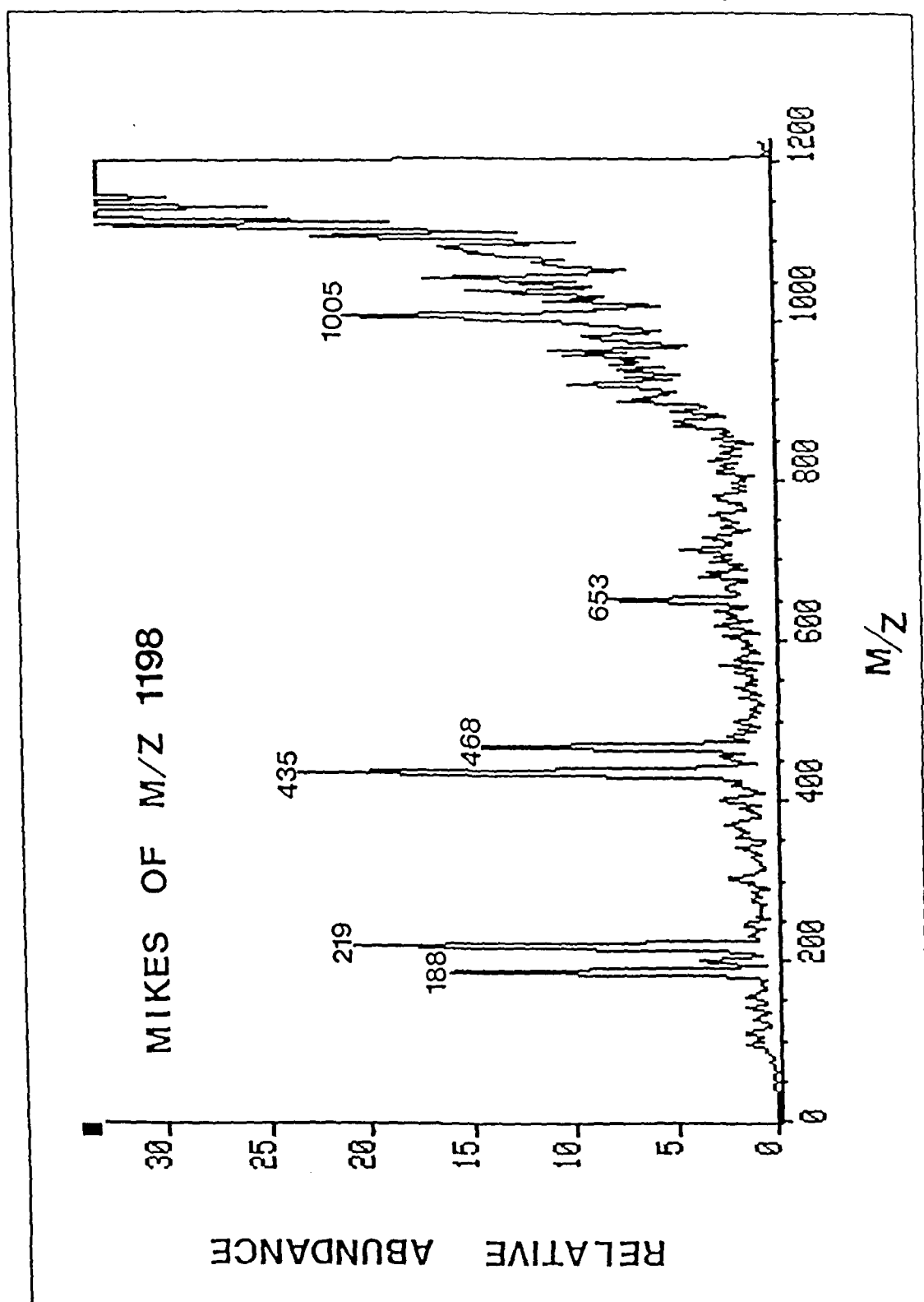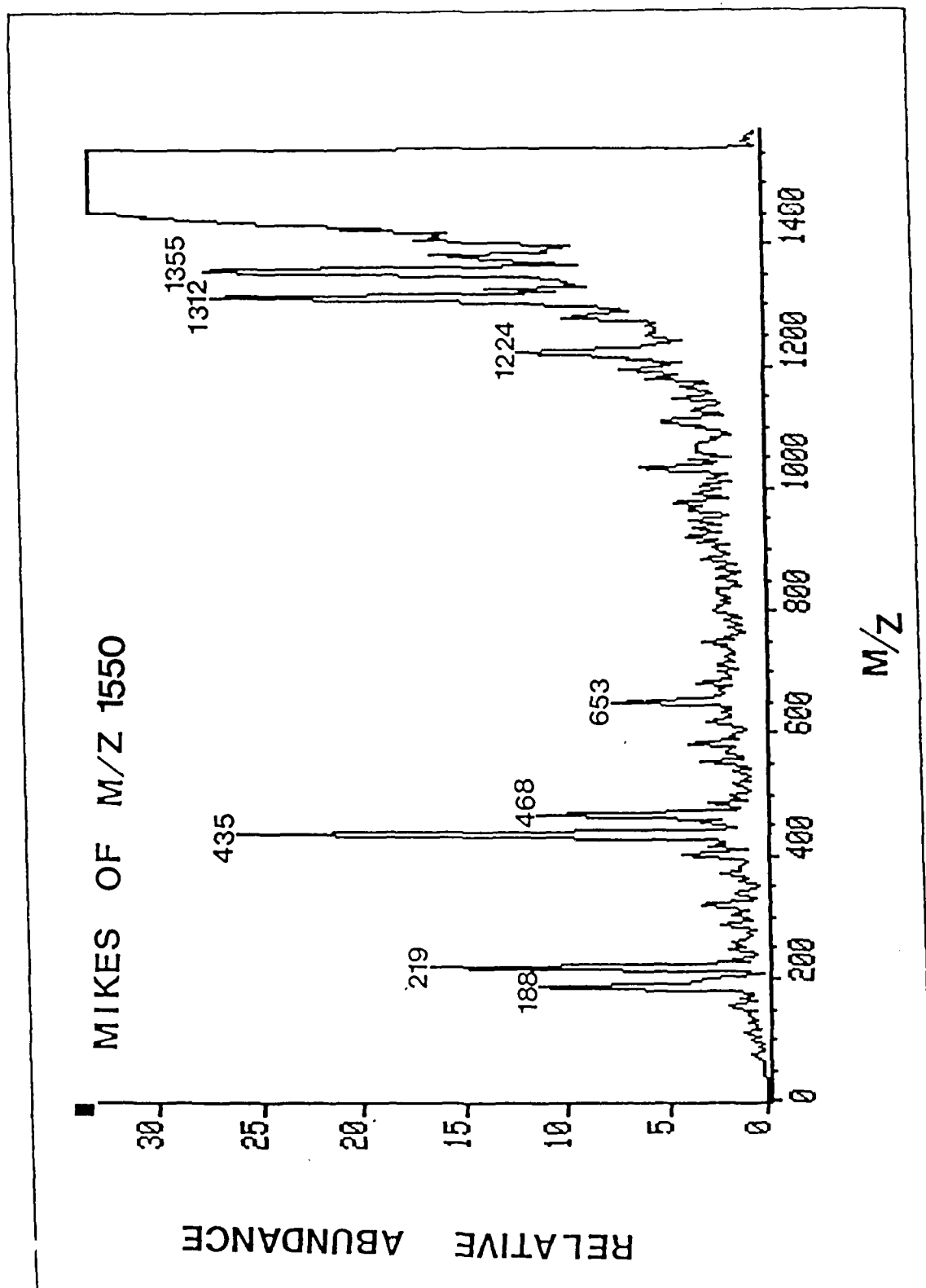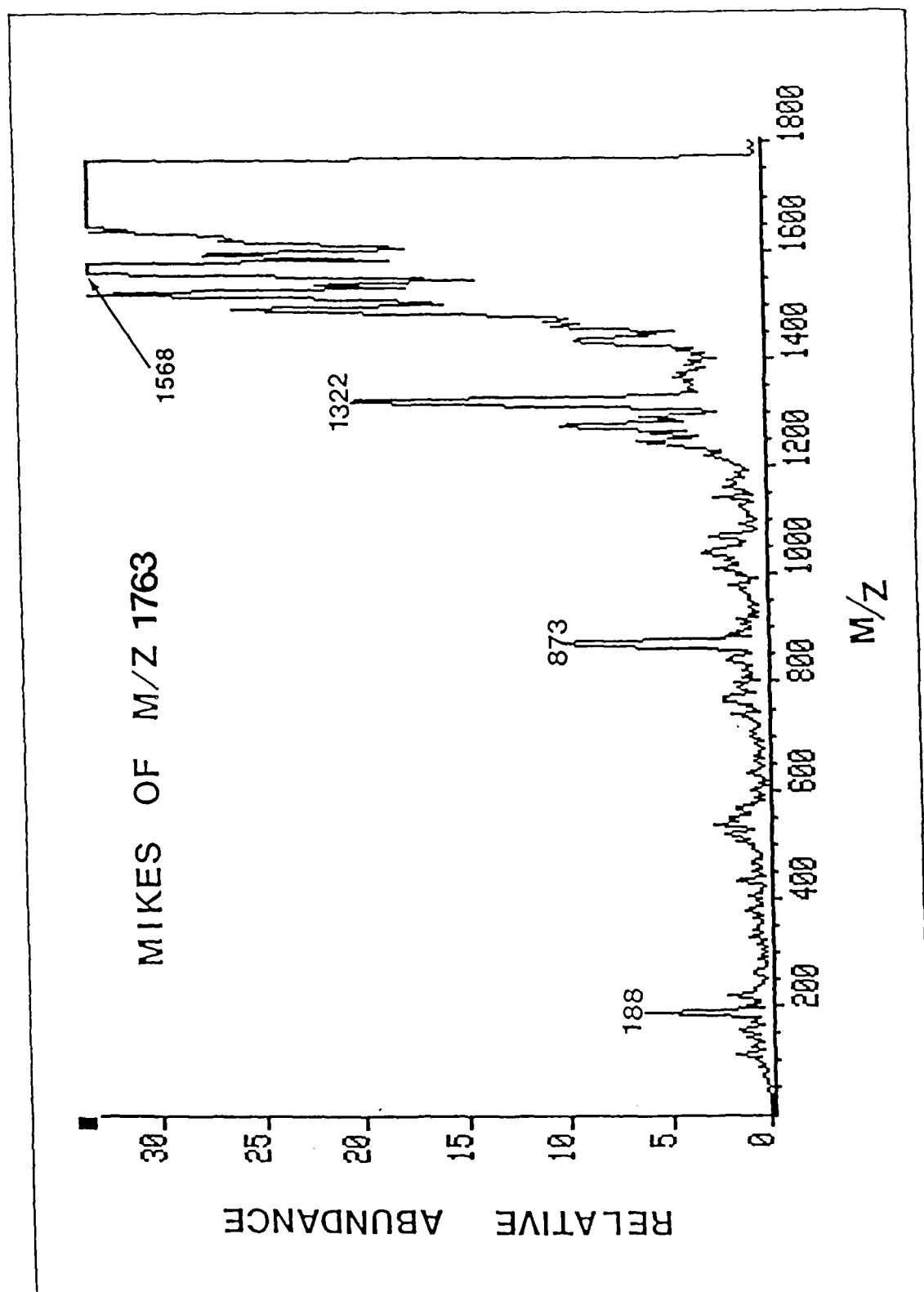
Figure 17

FAB—MS OF PERMETHYLATED LPSII
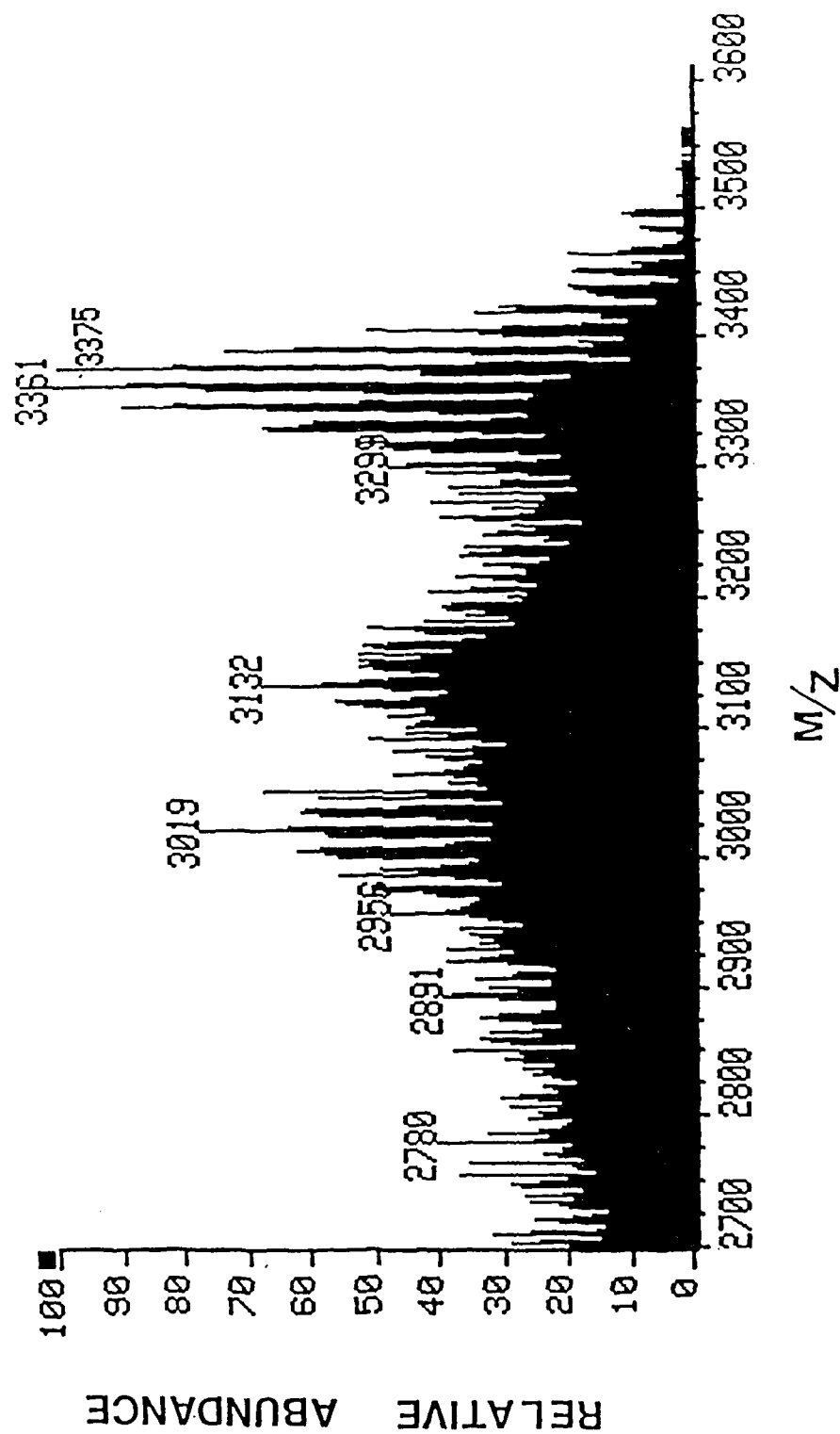
Figure 18

FAB—MS OF PERDUTEROMETHYLATED LPSII

Figure 19

DCI–MS OF PERMETHYLATED LPSII

Figure 20

DCI—MS OF PERDUTEROMETHYLATED LPSII

Figure 21

DCI-MS (NH3) PERMETHYLATED LPS-II
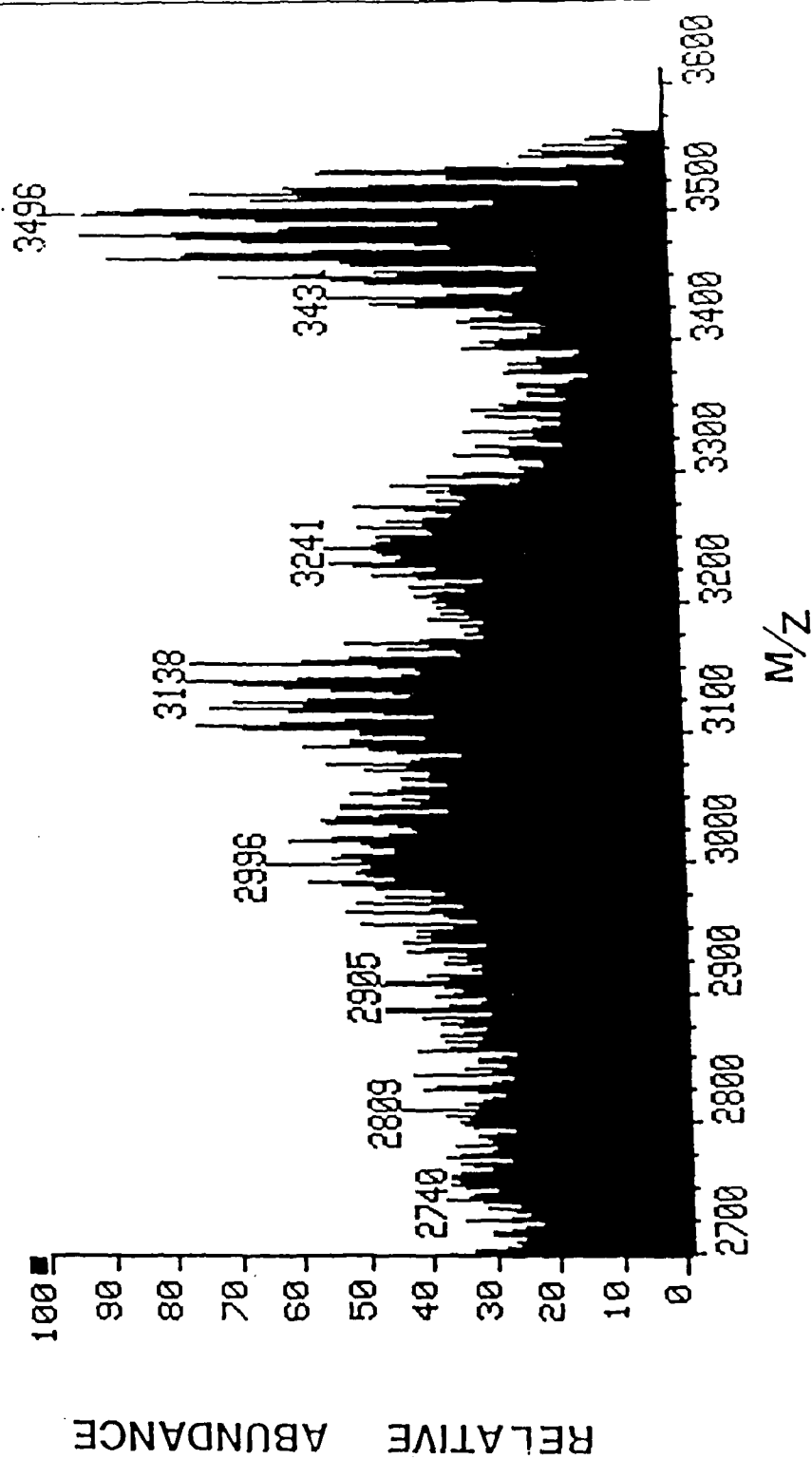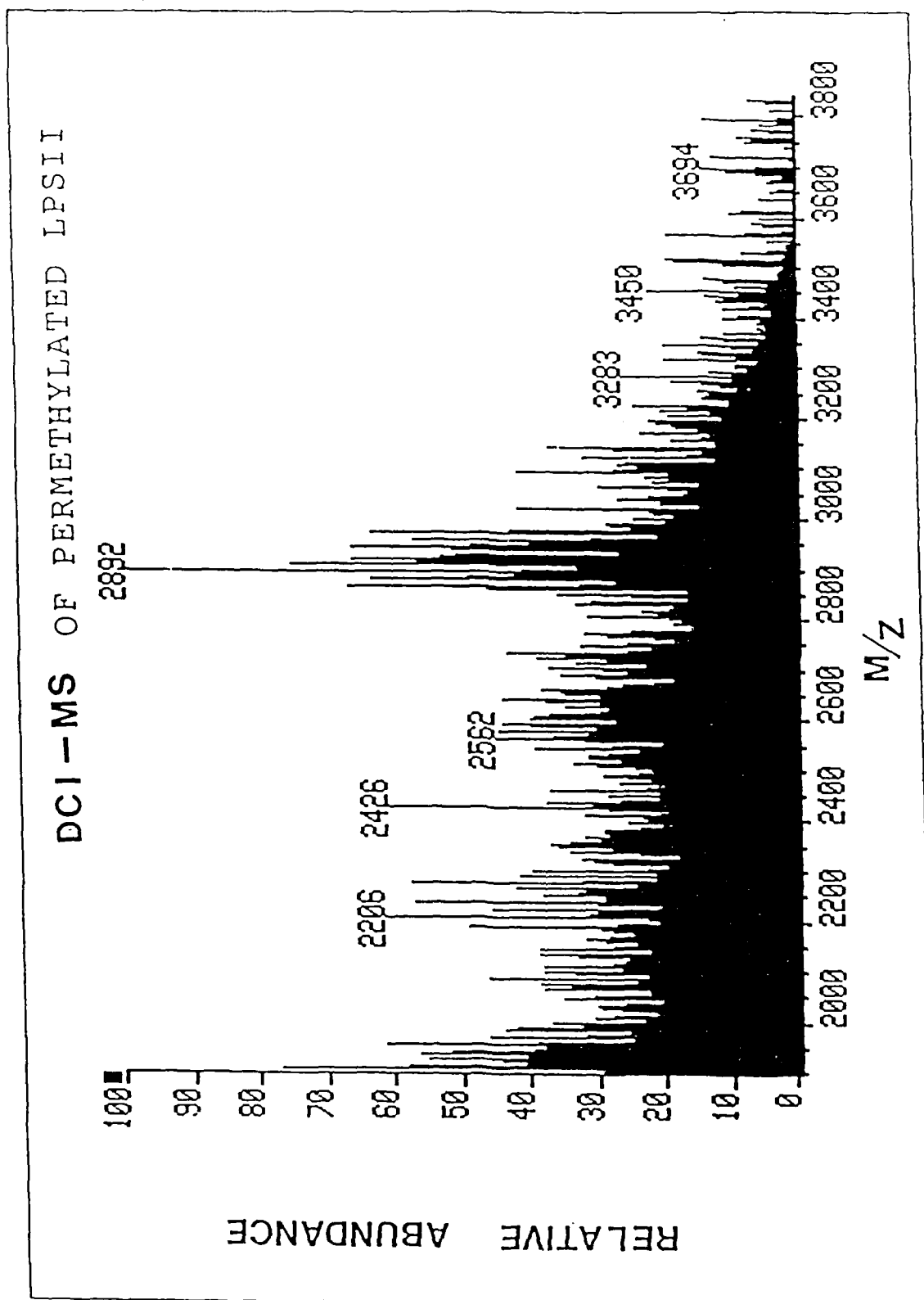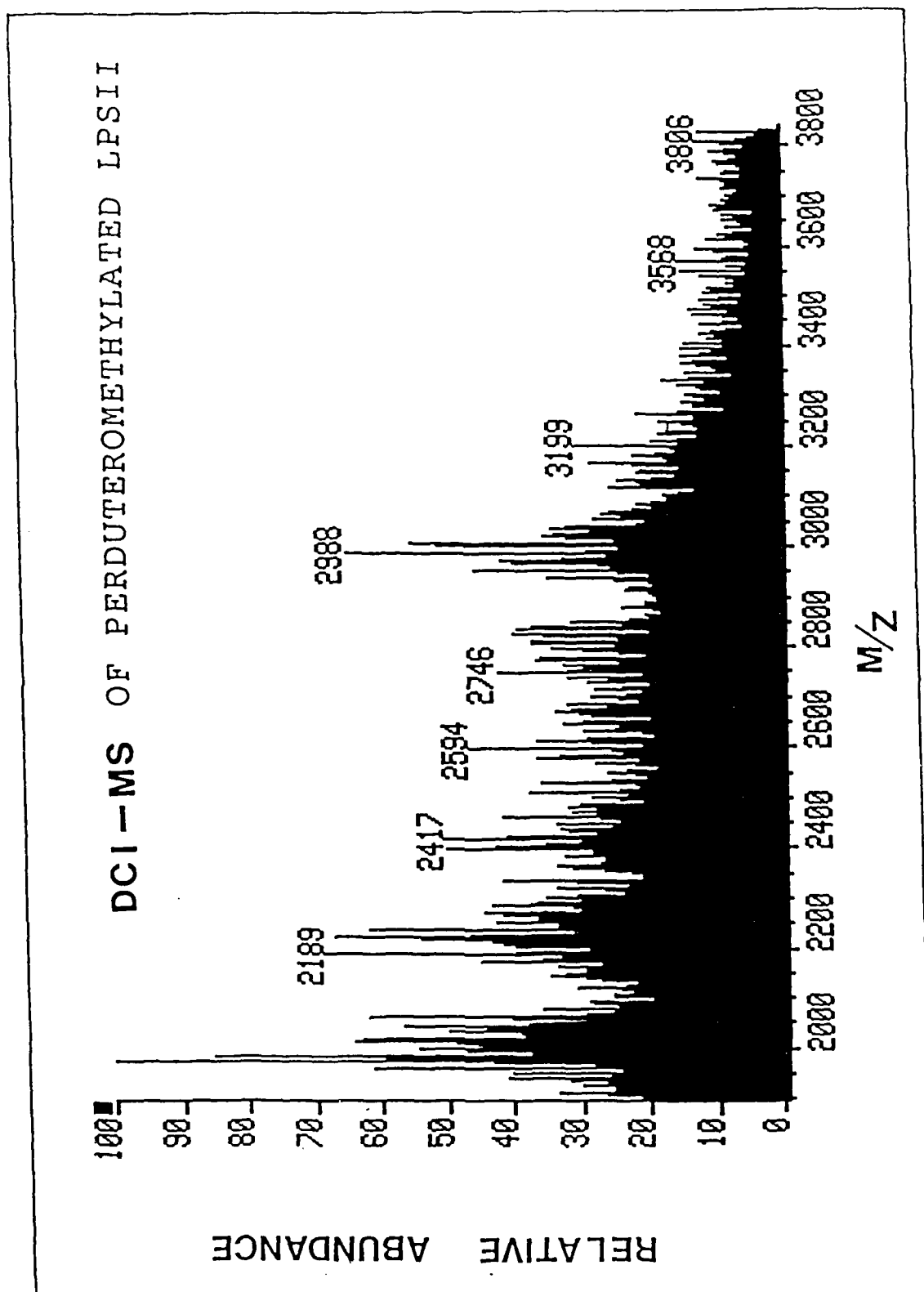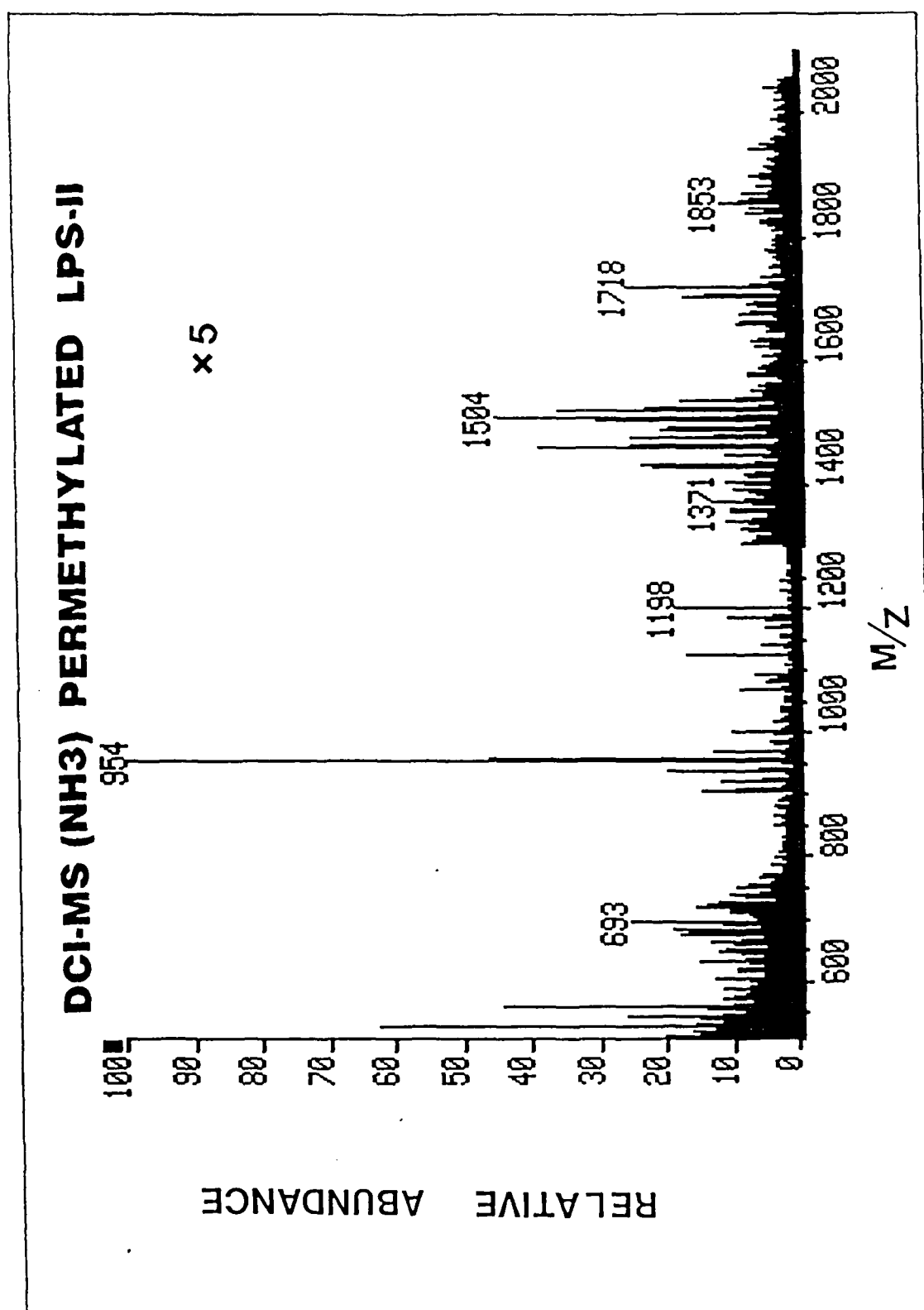
Figure 22

DCI MS (NH3) LPS 514 LIPID C
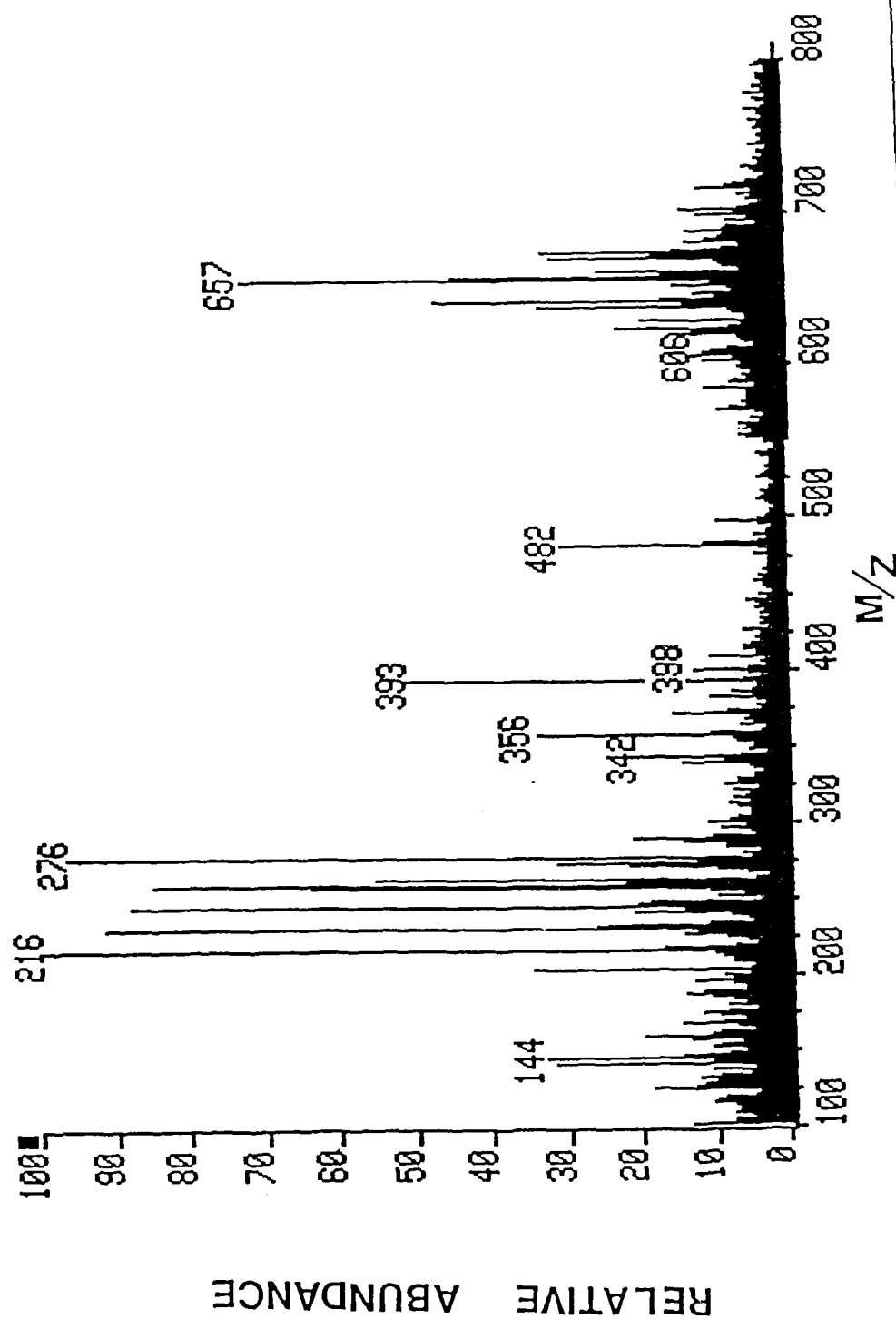
Figure 23

DCI MS (NH3) PER-O-ACETYL LPS 514 O-ANTIGEN
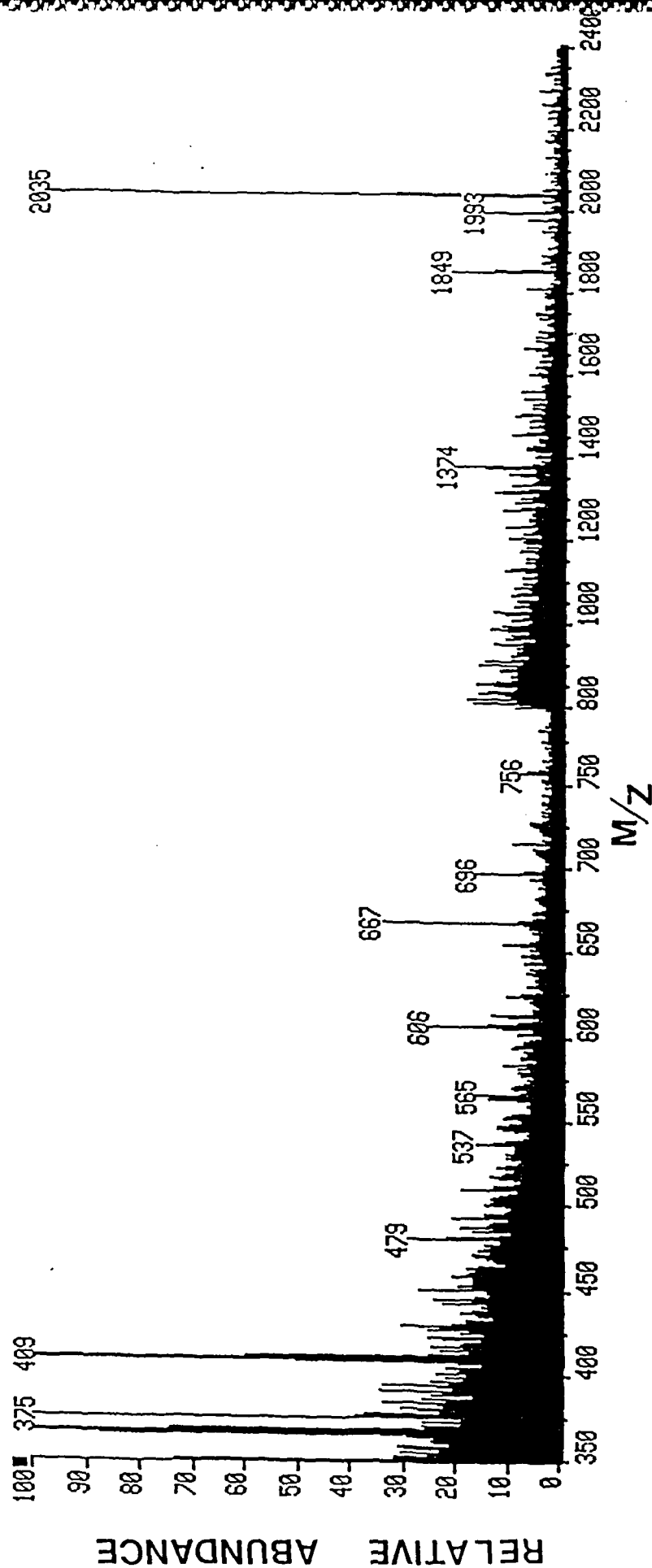
Figure 24

GAS CHROMATOGRAPHY IMF

YS CNE

SPLEEN BNE

Figure 25

GAS CHROMATOGRAPHY   IMF

CNE
Lot #1 E15

CNE E8

CNE
Lot #1 E7

Figure 26

GAS CHROMATOGRAPHY   IMF

Figure 27

GAS CHROMATOGRAPHY   IMF

Figure 28

FAB MS OF PER-O-ACYL COMPOUND X

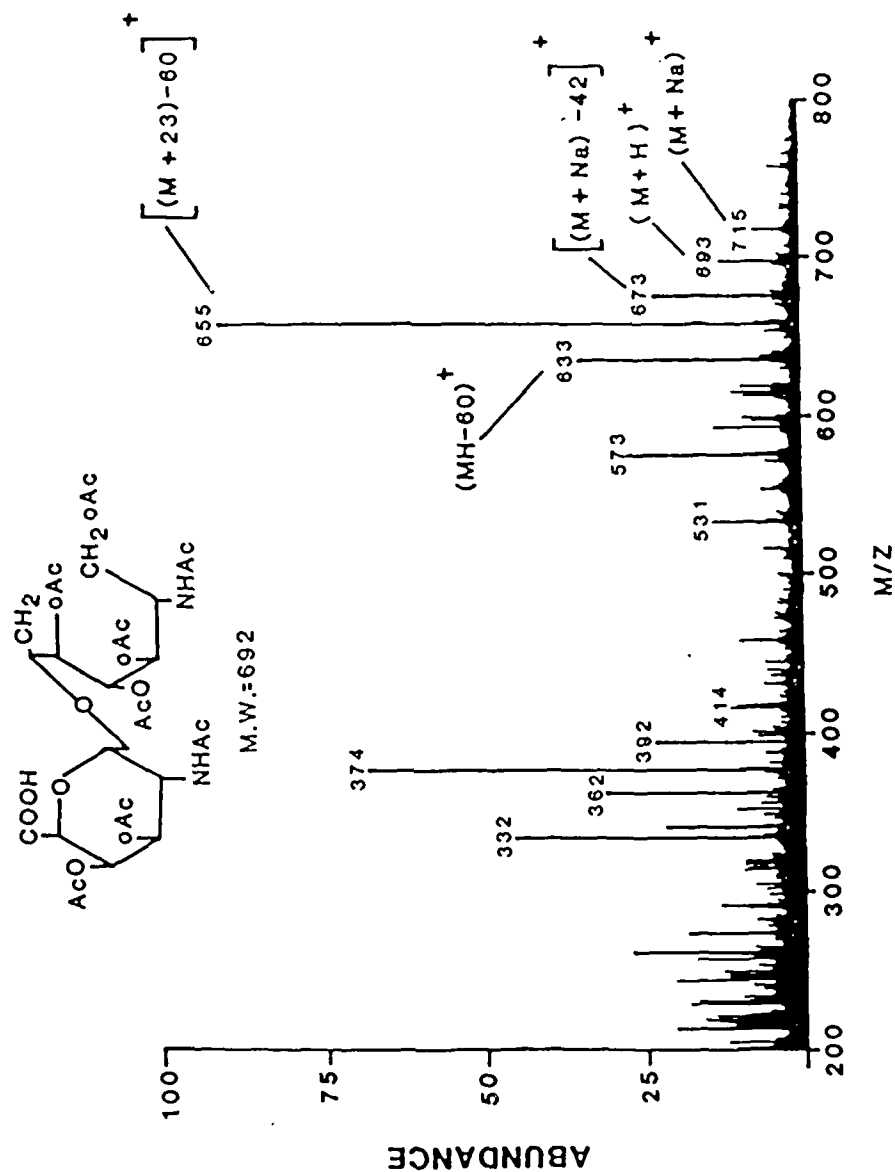Figure 29

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